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14. ABSTRACT
Our operating hypothesis is that *Checkpoint suppressor 1* (*CHES1*)/*FOXN3* is an androgen withdrawal-induced gene that promotes prostate cancer (PCa) resistance to apoptosis. The purposes of this research are two-fold. The first is to define the mechanisms of *CHES1* gene expression regulation and function, particularly in mediating apoptosis resistance during androgen ablation. Secondly, the tools yielded from our functional studies will be utilized to test the efficacy of *CHES1*-silencing therapy (CST) in preventing castration-resistant prostate cancer (CRPC) and to develop a mechanism-based noninvasive imaging strategy for monitoring the success of CST. Several significant findings were made. We defined the mechanisms through which CHES1 coordinates anti-apoptotic pathways, specifically enhanced PI3K/Akt activation and global regulation of genes negatively regulating apoptosis. We elucidated that CHES1-mediated AR repression diminishes amino acid-activated mTORC1, which consequently de-represses PI3K-Akt activity. We defined the precise mechanism of CHES1 as a direct transcriptional suppressor of pro-apoptotic *BNIP3* expression via its ability to recruit co-repressor complexes to the upstream regulatory region. Conversely, p53-mediated *CHES1* down-regulation is required for genotoxic stress to trigger apoptosis. Taken together, our findings provide strong support for exploiting CHES1 as a therapeutic target in that CHES1 antagonism would potentially lead to decreased anti-apoptotic PI3K-Akt signaling, combined reinstatement of pro-apoptotic gene expression (*i.e.*, *BNIP3*) and suppression of pro-survival genes, and reduced activity of oncogenic AR splice variants.

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INTRODUCTION

Androgen is a pivotal mediator of the growth, survival, and differentiation of prostate cancer (PCa) cells. Accordingly, androgen ablation is the first-line therapy for metastatic disease and dependably mediates disease regression. Unfortunately, this treatment is only palliative, as the disease typically recurs as castration-resistant prostate cancer (CRPC) approximately two years later and accounts for the 20% mortality rate due to this neoplasm. Therefore, defining the mechanisms underlying PCa survival during androgen withdrawal (AW) and the establishment of castration resistance are critical to enhancing our understanding of disease progression and the development of more efficacious therapies. We identified *FOXN3*/*CHES1* (*Checkpoint suppressor 1*) as a potential molecular mediator of PCa survival during androgen ablation. Our findings demonstrated that *CHES1* exhibits an AW-induced expression pattern and is an anti-apoptotic molecule that potentially acts via induction of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and/or down-regulation of the pro-apoptotic Bcl-2 family members *BNIP3* and *BAK1*. Importantly, antagonism of its function by RNA interference (RNAi)-mediated silencing resulted in apoptotic cell death of LNCaP cells selectively in the absence of androgen. That being said, ***the operating hypothesis of this work was that CHES1 is an AW-induced gene that functions to promote prostate cancer resistance to apoptosis and can be exploited as a therapeutic target.*** Therefore, there were two general purposes of this research. The first was to achieve a better understanding of mechanisms of *CHES1* gene expression regulation and function, particularly with respect to its role in mediating apoptosis resistance during androgen ablation. Secondly, the knowledge and tools yielded from our functional studies will be utilized to test the efficacy of *CHES1*-silencing therapy (CST) in preventing the emergence of CRPC and to develop a mechanism-based non-invasive imaging strategy for monitoring the success of the therapy.

BODY

This final report summarizes the research performed during the entire period of this award. At this point, I would also like to express our sincere gratitude to the Department of Defense for generously providing the funding for these studies. I enthusiastically writing this report based upon the results from the work performed. Our team has been able to confirm preliminary findings described in the original grant proposal and more importantly, significantly advance these along the lines of that described in our *Specific Aims* and the *Statement of Work*. These will be described below and in the *Supporting Data* section of this report.

Define the mechanism(s) through which CHES1 regulates apoptosis and acts as a dominant mediator of prostate cancer survival during androgen ablation.

--Clinical significance of *CHES1* expression

The studies proposed in this grant proposal were designed to define the function of CHES1/FOXN3 and provide *proof-of-principle* for exploiting it as a therapeutic target. Although evaluating CHES1 expression in clinical specimens was not proposed as a goal of this grant, we felt it was critical to validate its clinical significance. Towards this goal, we performed a bioinformatics-based study of existing, publically available datasets derived from microarray gene expression profiling of clinical specimens. *CHES1* expression in clinical prostate cancer samples was surveyed in a microarray dataset **(1)** using the Oncomine web application (https://www.oncomine.org/resource/login.html). CHES1 was down-regulated in 64% (16/25) of the samples relative to its expression in non-malignant prostatic epithelium **(Fig. 8A, 2011 Annual Report)**. Additionally, this analysis identified a 21-gene coexpression cluster composed of genes having ≥0.644 correlation with CHES1 **(Fig. 8B, 2011 Annual Report)**.

These findings extended our original findings in experimental models by revealing that CHES1 expression is altered in clinical PCa (*i.e.*, down-regulated) in the presence of androgen, and we interpret this finding to be consistent with our preliminary findings demonstrating its elevation in response to androgen ablation.

--Kinetics of *CHES1* and apoptosis-associated gene expression changes triggered by androgen ablation. **(Task 3)**

Our microarray profiling studies identified *CHES1*/*FOXN3* as an androgen withdrawal (AW)-induced gene in LNCaP cells. Subsequent experiments demonstrated that its expression was required for cell survival during androgen ablation and suggested that CHES1 function might antagonize apoptosis via the suppression of pro-apoptotic genes such as *BNIP3* and *BAK1*. The latter notion is certainly possible based upon its function as a transcriptional repressor **(2)**.

In an effort to better define resistance mechanisms operating during androgen ablation, it was critical to perform experiments specifically addressed the different types of this therapy, including, specifically *1)* androgen withdrawal (or deprivation; **Task 3a**) and *2)* combined androgen blockade (CAB; **Task 3b**) in which AW is combined with an anti-androgen to antagonize AR activity stimulated by residual androgens. Molecular and biochemical analyses by quantitative RT-PCR (qRT-PCR) and immunoblot analyses, respectively, were performed to evaluate the expression of *CHES1*, *BNIP3*, *BAK1*, and other molecules relevant to this context. After 96 hours of androgen deprivation, *CHES1* transcript expression increased 4.73-fold (relative to that found in the presence of 5α-dihydrotestosterone; DHT) and continued to climb to 18.2-fold at day 7 **(Fig. 1, 2010 Annual Report)**. The relevance of this phenomenon to tumor biology was demonstrated in CWR22 xenografts grown in intact or castrated nude mice, in which *CHES1* was up-regulated 6.15-fold at 14 days post-castration **(Fig. 2A, 2010 Annual Report**). In addition, *BNIP3* levels were reduced by 32-75% while *BAK1* steadily increased 4.88-fold **(Fig. 2B, 2010 Annual Report)**. Importantly, CHES1 protein levels also progressively increased following the shift to AW medium **(Fig. 3, 2010 Annual Report)**. To simulate CAB *in vitro*, LNCaP and CWR22Pc cells **(3)** were subjected to AW (3 days) and then treated with the antiandrogen bicalutamide (Casodex; CDX) **(Figs. 5 and 6, 2010 Annual Report)**. In both models, AR was highly expressed in the presence of DHT while CHES1 was only moderately expressed. As expected, AR was highly expressed in cells cultured in the presence of DHT while CHES1 was only moderately expressed. In contrast, high-dose CDX (25 mM) down-regulated the AR, which was accompanied by elevated CHES1 expression. In accord, there was an inverse association with BNIP3 expression under the same conditions.

As expected, AR levels were diminished during AW **(Fig. 4, 2010 Annual Report)**. Examination of apoptosis regulatory molecules demonstrated that BNIP3 protein levels exhibited were dramatically down-regulated **(Fig. 2B, 4, 2010 Annual Report)** and levels of Ser473-phosphorylated Akt were greatly increased. These results suggest the establishment of an anti-apoptotic cellular context.

--Development of model systems having conditional expression or silencing of *CHES1*/*FOXN3*.

One of the primary goals of this proposal is to better characterize the apoptotic process induced by inhibiting CHES1 function and to determine if suppression of CHES1 *in vivo* will delay the emergence of AI tumors. While the utilization of synthetic siRNA to transiently knock down CHES1 has provided excited results **(4)**, it can be difficult to dissect signaling mechanisms set against the dynamic background established by AW. Since this includes induced expression of endogenous *CHES1*, we felt the development of tetracycline (Tet)-inducible shRNA **(Tasks 2, 4, and 5)** and cDNA **(Tasks 1, 5)** expression models was central to

the success of this grant. These have will be discussed briefly and the new models are listed in the *Reportable Outcomes*.

----*Development of tetracycline-inducible CHES1 expression model systems.* A required component for this task was to also generate tetracycline-regulated LNCaP and CWR22Pc cell lines by retroviral gene transfer of pRevTet-on **(Task 5)**. Subsequently, these were infected with pLVX-Tight-Puro-FLAG-CHES1 lentivirus and selected with puromycin (1 mg/ml) in order to yield the new Tet-inducible FLAG epitope-tagged CHES1 expression models, LNCaP- and CWR22Pc-*tet*-FLAG-CHES1. In order to validate the models and address **Task 7**, a time-course experiment was performed to examine the kinetics and magnitude of Dox-inducible FLAG-CHES1 expression **(Fig. 1, 2011 Annual Report)**. As shown, FLAG-CHES1 expression was induced in a Dox-dependent manner and to a physiological level.

----*Development of tetracycline-inducible shRNA expression model systems.* This aspect of the project was quite successful. To accomplish this, **Tasks 2 and 4** were first performed in order to identify the most potent *CHES1*-specific hairpins (shRNAs) and then to move them into the tetracycline (Tet)/doxycycline (Dox)-inducible lentiviral expression vector pTRIPZ (Open Biosystems). The *CHES1* target sites and information for each shRNA construct are depicted in **Fig. 9** (2010 Annual Report). Constructs #1, 3, and 5 were validated by virtue of their ability to effectively repressed AW-induced CHES1 expression **(Fig. 10, 2010 Annual Report)** and were then utilized in the generation of the Tet-inducible shRNA constructs and LNCaP-*tet*-CHES1-Ri cell lines **(Task 5a)**, described in **Fig. 11** (2010 Annual Report). The establishment of stable cell lines was accomplished by **Task 5b**, and in which LNCaP-*tet*-CHES1-Ri-1 and -3 were validated for Dox-inducible repression of basal and AW-induced *CHES1* expression.

--Determine the position of CHES1 within the molecular hierarchy of the apoptosis regulatory network. **(Tasks 11, 14)**

A major goal of this proposal was addressed by this aim. One experimental approach we took to investigating this was to determine its functional position within the apoptosis regulatory network (Aim 1b), which also requires information gained from the other two sub-aims in Aim 1 as well as from Aim 2. While we originally viewed this as a "*molecular hierarchy*," we now know that CHES1 is integrated as a signaling node within a vast network and has multiple upstream regulators and functionally diverse downstream targets and effectors. I am very happy and enthusiastic to report that we have made very significant progress in this aspect of the grant and will summarize our findings in the following few paragraphs.

We showed that androgen is a critical regulator of signal transduction and survival signaling in that AW leads to diminished AR expression, persistent hyperactivation of the PI3K-Akt pathway, and decreased expression of the pro-apoptotic gene *BNIP3*. Subsequently, we demonstrated that CHES1 is a dominant mediator of these events, in that enforced CHES1 expression can recapitulate the events triggered by androgen deprivation, in part through its repression of AR activity **(Fig. 4, 2011 Annual Report)**. Furthermore, we demonstrated that mTOR complex 1 (mTORC1) is a critical integration point for the survival response to decreased AR signaling and elucidated the molecular connection between AW and Akt hyperactivation **(Fig. 2A, 2011 Annual Report)**. Specifically, the results demonstrated that mTORC1 activation, as evidenced by S6K1(T389) phosphorylation, is highly dependent upon androgen (*e.g.*, R1881) and that Akt hyperactivation results from AW-induced mTORC1 inactivation, which consequently de-represses the PI3K-Akt pathway. This was further supported by the fact that specific inhibition of mTORC1 inhibition with rapamycin resulted in elevated phospho-Akt (S473) levels similarly to that of AW. Subsequently, we demonstrated

that androgen predominantly regulates mTORC1 activation in response to amino acid (AA) signaling since AA-stimulated S6K1(T389) phosphorylation only occurred in the presence of androgen and was completely abolished during AW **(Fig. 1B, 2012 Annual Report)**. In summary, these results demonstrated that AW- and CHES-mediated survival signaling in PCa is mediated via mTORC1 and integrates with metabolism.

Amino acid signaling to mTORC1 has been demonstrated to proceed via an interaction between mTOR-raptor (mTORC1) and Rag GTPase heterodimers and their subsequent recruitment to the surface of the lysosome **(5,6)**. Our findings presented in the *2011 Annual Report* demonstrated that AW reduced the interaction of Rag heterodimers with raptor in hormone-sensitive cells **(Fig. 3B, 2011 Annual Report)** and that enforced expression of CHES1 was a dominant mediator of this event **(Fig. 3C, 2011 Annual Report)**. We then provided further evidence for this mechanism by demonstrating that the requirement for androgen can be bypassed by enforced expression of a RagB mutant mimicking the GTP-bound conformation ($RagB^{GTP}$) in that LNCaP cells stably expressing $RagB^{GTP}$ (LNCaP-FLAG-Rag^{GTP}) exhibited androgen-independent, or insensitive, interactions between mTOR and RagB **(Fig. 2C, 2012 Annual Report)**. Moreover, enforced $RagB^{GTP}$ expression overcame CHES1-mediated suppression of mTORC1 interaction with RagB/D heterodimers **(Fig. 2D, 2012 Annual Report)**. Since mTORC1 displayed strong androgen-dependence, we hypothesized that constitutive activation of $RagB^{GTP}$ would confer androgen independence upon stably expressing LNCaP sublines. However, we observed that enforced activation of androgen- and amino acid-regulated mTORC1 compromised the survival of LNCaP cells in the absence of androgen in that LNCaP-$RagB^{GTP}$ cells exhibited a high percentage of apoptosis after extended times in culture **(Fig. 3, 2012 Annual Report)**. While this seemed paradoxical at first, we reason that persistent amino acid signaling to mTORC1 in the absence of androgen might be interpreted as inappropriate signaling, which leads to an overall imbalance in signaling.

While our data has demonstrated that mTORC1 activation in LNCaP cells is strongly androgen-dependent, we have noted that a low level of S6K1(T389) phosphorylation always remains even upon extended periods of androgen deprivation **(Fig. 1A,** *lane AW-7d*; **2012 Annual Report)**. We demonstrated that this residual mTORC1 activity was derived from growth factor signaling since polypeptide growth factors such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) were full capable of stimulating S6K1(T389) phosphorylation in the absence of androgen and complete absence of serum **(Fig. 4A, Annual Report)**. These findings were reinforced in a complimentary experiment that demonstrated that low levels of S6K1(T389) phosphorylation were reinstated rapidly after removal of a PI3K inhibitor (LY294002), which completely inhibited growth factor signaling to mTORC1 **(Fig. 4B, Annual Report)**. In contrast, full activation of TORC1 required exposure to androgen for 16-24 hours.

In the past year, we extended these studies to investigate the role of this AR-mTORC1-Akt signaling axis in the establishment of castration resistance. Our results demonstrated that disruption of this axis represents a critical molecular event in the transition to CRPC since PI3K-Akt levels are markedly elevated in CRPC LNCaP-cds sublines despite restoration of AR signaling **(Figs. 1, 3)**. Interestingly, androgen-responsive mTORC1 induction is retained in AR-expressing CRPC cell lines **(Fig. 1)**. LNCaP cells are acutely hyperdependent on PI3K for survival during AW and are sensitive to clinically-relevant agents, such as Adr, arsenic trioxide, and GCP, which induced apoptosis by performing dual actions as mimetics of androgen ablation (AR degradation) and signal transduction inhibitors (mTORC1 inhibition)**(Fig. 3)**. In contrast, LNCaP-cds cell lines were remarkably resistant to these treatments, despite p53 induction and AR degradation. Further studies revealed that PI3K-Akt signaling was refractory

to LY294002 and implicated Bcl-2 overexpression as the downstream mechanism conferring pan-resistance to multiple apoptotic stimuli **(Fig. 3),** and especially since restoration of pro-apoptotic downstream from Akt was unable to mediate apoptosis **(Fig. 4)**. This notion was confirmed by the ability of the Bcl-2 inhibitor HA14-1 to sensitize LNCaP-cds cells to Adriamycin **(Fig. 5)**. In summary, our results suggest that 1) the progression of CaP to CRPC is mediated via the transient de-repression and eventual loss of AR-mediated negative regulation of anti-apoptotic mechanisms.

-- CHES1-mediated regulation of androgen receptor activity **(Task 14)**

Another major advancement was the finding that CHES1 both physically interacts with and represses the activity of the AR. Based upon findings that other members of the forkhead family can directly interact with the AR **(7,8)**, we hypothesized that *CHES1*/*FOXN3* might also. In order to investigate this, we performed standard co-transfection experiments with FLAG-tagged CHES1 and different forms of HA-tagged AR, followed by immunoprecipitation-immunoblot analysis for the presence of FLAG-CHES1 in the immune complexes. Four forms of the AR were used: full-length wild-type AR (ARwt), C-terminal truncation mutants containing only the N-transactivation domain (AR/NTD) or NTD plus nuclear-localization signal (AR/NTD-NLS), and the clinically-relevant splice variant AR3 **(9)**. As shown in **Figure 4A** (2011 Annual Report). CHES1 interacted with full-length wild-type AR and all truncated variants tested. Interestingly, reporter assays conducted in LNCaP cells demonstrated that enforced expression of CHES1 repressed transactivation of the *PSA* promoter by full-length, wild-type AR **(Fig. 4B, 2011 Annual Report)**. In contrast, CHES1 had no inhibitory effect upon C-terminally truncated variants **(Fig. 4C, 2011 Annual Report)**. Taken together, the results suggest that down-regulation of AR during androgen ablation is due to several collaborating mechanisms: removal of ligand (DHT), mTORC1-mediated down-regulation of expression, and CHES1-mediated repression of its transcriptional activity. Moreover, truncated AR variants are not influenced by CHES1-mediated AR repression, thereby implicating this as a mechanism through which Pca cells can escape androgen ablation.

-- Determine if repression of *CHES1* expression is a critical event in p53-mediated apoptosis. **(Tasks 6, 21)**

While we have demonstrated that CHES1 is a dominant mediator of anti-apoptotic signaling (*e.g.*, PI3K-Akt hyperactivation, *BNIP3* suppression), we conversely hypothesized that its down-regulation would be necessary for a pro-apoptotic signal to be successfully propagated in response to inducers of genotoxic stress, including ionizing radiation (IR), mitomycin C (MMC), and adriamycin (Adr). Our initial studies with IR demonstrated that *CHES1* was rapidly and potently repressed, and that this occurred coincidently with p53 activation and transcriptional induction of CDKN1A **(4)**. Having identified a half-site for p53 binding identified within the *CHES1* proximal promoter provided further support for *CHES1* transcriptional repression as being an immediate-early response to p53 activation. This was validated by performing co-transfection dual-luciferase reporter assays with our pGL3B-*CHES1*-RR1/3.5 reporter **(Task 13)(Fig. 6A, 2011 Annual Report)** and different p53 expression constructs**.** These demonstrated that wild-type p53 potently repressed *CHES1* promoter activity (*i.e.*, 77% of control) while the p53 loss-of-function and gain-of-function mutants either increased activity (G245S) or had little or no effect **(Fig. 6C, 2011 Annual Report)**.

We pursued these studies further using the chemotherapeutic agents MMC and Adr, which are known to induce DNA damage and a potent p53 response. The association between p53 induction, CHES1 repression, and apoptosis of LNCaP cells was nicely

demonstrated in the MMC dose-response and time-course experiments depicted in **Figs. 7 and 8** (2010 Annual Report). In addition, markers indicative of p53 activation [phospho-p53 (Ser15), MDM2) and apoptosis (*e.g.*, PARP cleavage) were elevated. Although the p53 pathway was apparently engaged, CHES1 levels were not reduced. Consistent with the survival-promoting mechanisms described for CHES1 above, CHES1 down-regulation was accompanied by a reduction in phospho-Ak(S473) levels and increased BNIP3 expression **(Fig. 5A, 2011 Annual Report)**. Importantly, this shift toward a pro-apoptotic manifested in the induction of apoptosis as indicated by PARP cleavage. In contrast, apoptosis was not observed in CWR22Pc cells, as indicated by a lack of PARP cleavage **(Fig. 7, 2010 Annual Report)**. Using an siRNA approach **(Task 21a)**, it was further demonstrated that silencing of p53 markedly diminished both its basal expression and induction by Adr (1 μg/ml, 4 hours), which consequently resulted in stabilization of CHES1 levels **(Fig. 5C, 2011 Annual Report), a**nd abrogation of Adr-mediated apoptosis, as evidenced by the absence of PARP cleavage **(Fig. 5, 2012 Annual Report)**. In conclusion, the results demonstrated that repression of CHES1 is mediated via p53 and is a critical event in chemotherapy-induced apoptosis.

Define the functional properties of CHES1 as an anti-apoptotic transcription factor.

--Define the CHES1 expression profile using Affymetrix-based microarray profiling. **(Task 10)**

Since CHES1/FOXN3 is a member of the Forkhead box (FOX) family of transcription factors, another vital goal of this grant was to further define the properties of CHES1 by characterizing its function as an anti-apoptotic transcription factor. In this regard, a major point of focus has been directed upon the pro-apoptotic gene *BNIP3* as a target of repression (*discussed below*). At the same time, we hypothesized that CHES1 has a more global influence upon PCa biology during androgen ablation through its capacity to shape the transcriptome via its action on numerous other direct and indirect target genes. In order to identify the complete cohort of CHES1-regulated genes, we performed microarray-based genome-wide gene expression profiling with Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays in order to identify the genes whose expression were differentially expressed in LNCaP-*tet*-FLAG-CHES1 cells engineered for conditional overexpression of *CHES1* relative to the vector-control cell line **(Fig. 6, 2012 Annual Report)**. In this experiment, *CHES1* was induced in two separate clones by treatment of the cells with doxycycline (100 μg/ml) for 24 and 48 hours. The results demonstrated that CHES1/FOXN3 is indeed a global regulator of transcription in that there were a total of 3,401 genes exhibiting ≥1.5-fold change in expression (1,902 up-regulated and 1,499 down-regulated). Hierarchical clustering of the data was performed and the results depicted as a heatmap **(Fig. 6, 2012 Annual Report)**. Since CHES1 overexpression also mediates AR down-regulation, a significant subset of CHES1-regulated genes overlaps with genes comprising the AR transcriptome.

--Determine if CHES1 increases resistance to apoptosis by functioning as a transcriptional repressor of the pro-apoptotic gene *BNIP3*. **(Task 16)**

Our findings suggest that a critical, pro-survival activity of CHES1 is to function as a direct, transcriptional repressor of the pro-apoptotic gene *BNIP3* (*BCL2/adenovirus E1B 19 kd-interacting protein 3*). Using the pGL3B-*BNIP3*(4.0) construct **(Task 13)**, dual luciferase reporter assays demonstrated that *BNIP3* promoter activity was markedly reduced during androgen withdrawal (63% decrease) and when co-transfected with CHES1 expression constructs (73-89% decrease) **(Fig. 7, 2011 Annual Report)**. Moreover, the ability of CHES1 to interact with components of transcriptional co-repressor complexes has been demonstrated

previously **(10)** and in our studies, we definitively demonstrated that CHES1 is a *bona fide* transcriptional repressor of *BNIP3* via assembly of a co-repressor complex at this locus.

For this, a series of experiments utilizing chromatin immunoprecipitation (ChIP) assays were performed in order to define the assembly and function of a CHES1 co-repressor complex at different regions (*labeled A-E*) across the *BNIP3* upstream regulatory region **(Fig 7A, 2012 Annual Report)**. These were selected based upon *1)* bioinformatics analysis of the *BNIP3* enhancer/promoter for putative forkhead-binding sites (*i.e.*, regions A, C, D) and *2)* the results of ChIP-on-chip analysis (*discussed below*), which identified a CHES1-binding site approximately 1,605-bp upstream of the *BNIP3* transcription start site **(Table 1, 2012 Annual Report, Sequence ID# 42,)**. This is represented by "*region D*" and contains three forkhead binding sites within 30-bp. Initial ChIP assay experiments with LNCaP cells were performed to validate CHES1 binding to this this site and to evaluate the other regions. These demonstrated marked elevation in CHES1 binding to regions A and D after androgen-deprivation (96 hours) as compared to that in the presence of androgen (4.77- and 10.37-fold respectively) **(Fig. 7B, 2012 Annual Report)**. Time-course experiments to study the kinetics of CHES1 binding to region D and demonstrated that increased recruitment was evident after 24 hours, peaked at 72 hours, and persisted through 96 hours after androgen deprivation **(Fig. 7C, 2012 Annual Report)**.

In order to determine that CHES1 was indeed participating in the assembly of a functional co-repressor complex at the *BNIP3* enhancer/promoter, we also evaluated the co-recruitment of the Sin3A co-repressor and histone deacetylase 1 (HDAC1). The functional consequences of complex formation were assessed by monitoring the levels of acetylated histone H3 (Acetyl-H3) and H4 (Acetyl-H4), as well as the presence of RNA polymerase II (RNA pol II). These experiments were performed with the tetracycline-inducible LNCaP-*tet*-FLAG-CHES1 **(Task 5)** cell line in order to have better control over CHES1 activation (*i.e.*, by adding doxycycline to the medium). The results demonstrated that the CHES1 co-repressor complex assembled at regions A and D, but much more robustly at the latter. This was evidenced by heightened enrichment of CHES1 (15.73-fold) in conjunction with Sin3A and HDAC1 (5.70- and 7.70-fold, respectively) and a concomitant reduction in the levels of acetylated histone H3 and H4 that were associated with the chromatin at that locus **(Fig. 8, 2012 Annual Report)**. In addition, RNA polymerase II binding was reduced at the proximal promoter and transcription start site (region E). Taken together, CHES1 suppresses *BNIP3* expression via the recruitment of a co-repressor complex, which leads to epigenetic modifications in favor of a closed chromatin conformation and decreased accessibility of RNA polymerase II to initiate transcription.

--Characterize the genome-wide targets of CHES1-mediated chromatin remodeling. **(Task 24)**

In order to gain a more comprehensive understanding of how CHES1 functions as a transcriptional regulator to impact molecular and biological processes within the cell, we performed ChIP-on-chip and ChIP-Sequencing (ChIP-Seq) analyses in order to identify the complete set of target genes that CHES1 binds directly to. For this, CHES1-associated chromatin DNA was precipitated in a ChIP experiment similar to that described above in **Fig. 8** (2012 Annual Report), but instead of analyzing the immunoprecipitated DNA for only selected regions of interest, ChIP-on-chip analysis applies the DNA ("ChIP") to tiling microarrays ("chip"; Affymetrix GeneChip Human Promoter 1.0R Array) in order to perform a genome-wide scan for all CHES1-binding sites represented by the CHIP DNA. Peak detection and false discovery rate (FDR) computation were performed using CisGenome software **(11,12)** and the assay revealed a novel set of 409 genome-wide recruitment sites for CHES1; the sites having the highest enrichment are presented in **Table 1** (2012 Annual Report). This information was then translated into the identification of potential CHES1 target genes by the determination of the

closest genomic locus to each of these sites, as well as their locations relative to key functional regions (*e.g.*, UTR, exon). As discussed above, CHES1 binding to the *BNIP3* enhancer/promoter was independently detected with this approach (Sequence ID #42, -1,605 bp to TSS). In order to determine if CHES1 target genes are biologically- and/or functionally-related, gene ontology (GO) analysis was performed **(Table 2, 2012 Annual Report)** using the Gene Functional Classification Tool available from DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp). Consistent with our previous findings, GO analysis highlighted the function of CHES1 in the coordinated regulation of genes that control apoptosis. For instance, this revealed enrichment for multiple sets of genes that function in various biological processes, including negative regulation of apoptosis, nitrogen compound metabolism, and chromatin/chromosome organization. As an additional measure to identify the most significant biological processes based upon GO classifications and in an unbiased manner, we performed functional annotation clustering and this underscored that overlapping sets of CHES1 target genes are markedly enriched for those functioning in pathways that regulate apoptosis **(Fig. 9, 2012 Annual Report)**. Initially, this experiment was also repeated using ChIP-Seq analysis in which the ChIP DNA samples (and corresponding Input DNA) are directly sequenced. For this, sequencing "libraries" were prepared followed by multiplex sequencing (50bp, single-read, 8 samples per lane) on an Illumina HiSeq 2000. Subsequently, the DNA sequences (~25^6 reads/sample) were mapped to the human genome reference sequence (hg19) using Bowtie 2 **(13)** and then peak detection performed using Model-based Analysis of ChIP-Seq (MACS) **(14)**. Although ChIP-Seq results demonstrated CHES1 recruitment to the *BNIP3* locus, this was only modest, ad in general, this approach unexpectedly was not as robust as ChIP-on-Chip in that we did not observe many CHES1 recruitment sites. As a result, we feel that the ChIP-on-Chip results are more informative and used for moving forward.

Determine if adjuvant antagonism of CHES1 during androgen ablation will prevent the emergence of castration-resistant prostate cancer (CRPC) (Tasks 12, 15, 19, 22)

The primary goals of **Aim 3** are *1)* to provide *proof-of-principle* evidence that antagonism of CHES1 function with *CHES1*-silencing therapy (CST) can prevent the emergence of castration-resistant tumors in an animal model and *2)* to test a mechanism-based noninvasive imaging strategy for monitoring the success of CST. The necessary components for these experiments were established and include LNCaP and CWR22 sublines that have tetracycline-inducible expression of *CHES1*-specific shRNA (CHES1-Ri) **(Task 12)** and double-stable LNCaP and CWR22 sublines infected with inducible *CHES1*-Ri expression and *BNIP3* reporter constructs, designated as LNCaP- and CWR22-*tet*-CHES1-Ri/BNIP3-*Rlu*, respectively **(Task 19)**. The former were reported in the *2011 Annual Report* and the double stable cell lines were established during the current year. The completion of the *in vivo* mouse studies **(Tasks 15, 22)** were substantially hampered due to obtaining very poor rates of tumor take/establishment of LNCaP-based tumors in athymic nude mice (*e.g.*, 2 of 12 mice injected). However, having established the LNCaP-*tet*-CHES1-Ri cell lines as xenografts **(2011 Annual Report)**, we were able to validate the use of these as models for CST **(Fig. 11, 2012 Annual Report)**. For this, LNCaP-*tet*-CHES1-Ri tumors were established in nude athymic mice. Bilateral orchiectomy was then performed in order to simulate androgen deprivation and to induce endogenous CHES1 expression. CST was then started by feeding the mice doxycycline (250 mg/ml) in their drinking water to induce expression of the *CHES1*-specific shRNA. Immunoblot analysis demonstrated that CHES1 expression was markedly suppressed, which was accompanied by the consequential de-repression of pro-apoptotic BNIP3 expression and decreased levels of

Akt(S473) phosphorylation. In summary, although we could not complete the experiments involving combined CST and noninvasive monitoring, we were able to accomplish the proof-of-principle experiments that confirmed the concept of CST as being a viable approach to enhancing the efficacy of androgen ablation.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed that induction of CHES1/FOXN3 expression (transcript and protein) is a general feature of androgen ablation in multiple prostate cancer models *in vitro* and *in vivo* and that combined androgen blockade induces CHES1 expression more highly than androgen deprivation alone.
- Gained insight into the clinical significance of CHES1 expression in that it is a gene normally expressed in non-malignant prostatic epithelium, but markedly decreased in primary prostate cancers.
- Demonstrated that CHES1 functions as a dominant mediator of androgen withdrawal-induced survival signaling by suppressing amino acid signaling to mTORC1, which in turn, leads to de-repression and hyperactivation of the PI3K/Akt signaling pathway. Moreover, the AR-CHES1-PI3K/Akt signaling axis is aberrantly regulated in CRPC cells.
- Discovered that CHES1 represses mTORC1 activation by inhibiting its association with Rag GTPases and targeting to the surface of the lysosome.
- Demonstrated that CHES1 down-regulation is required for chemotherapy-induced apoptosis and is mediated by p53-mediated transcriptional repression of CHES1 expression.
- Confirmed that *BNIP3* is a direct target of transcriptional repression by CHES1 and elucidated the mechanism to involve CHES1-mediated assembly of a co-repressor complex containing Sin3A and HDAC1 in the *BNIP3* proximal enhancer/promoter region, which consequently mediated epigenetic changes (*i.e.*, histone H3 and H4 deacetylation) that reduced accessibility to RNA polymerase II.
- Comprehensively defined the CHES1 transcriptome (gene expression profile) and cistrome (genomic recruitment sites), which provided tremendous insight into the global and dominant impact of its function upon biological processes that are critical to mediating prostate cancer progression, including the negative regulation of apoptosis.
- Validated the LNCaP-*tet*-CHES1-Ri conditionally-expressing *CHES1* shRNA cell lines as models for utilization in *proof-of-principle in vivo* studies of CHES1-silencing therapy.

REPORTABLE OUTCOMES

Manuscripts

1. Mudryj, M., and Tepper, C.G. (2013). On the origins of the androgen receptor low molecular weight species. Hormones and Cancer, 4(5): 259-269.
2. Xiang, N., Yang, J.K., Webb, J.W., Wee, C.B., Boucher, D.L., Baron, C.A., Scott, S.C., Shi, X.B., de Vere White, R.W., Gregg, J.P., Kung, H.J., and Tepper, C.G. (2012). Identification of checkpoint suppressor 1 (CHES1) as an androgen-repressed gene mediating neuroendocrine differentiation and survival of prostate cancer cells. Cancer Res *In preparation*.
3. Xiang, N., Wee, C.B., Boucher, D.L., Ali, H.M., Liu, S.Y., Albert, S., Shi, X.B., Gregg, J.P., de Vere White, R.W., Kung, H.J., and Tepper, C.G. Mammalian target of rapamycin as an integrator of androgen withdrawal-induced survival mechanisms. J Biol Chem *In preparation*.

Abstracts and Presentations

1. Xiang, N., Ali, H.M., Davis, R.R., Liu, S.Y., Boucher, D.L., Gregg, J.P., Kung, H.J., and Tepper, C.G. Potential role of *CHES1*/*FOXN3* as an anti-apoptotic regulator of prostate cancer response to androgen ablative and genotoxic therapies. Poster presented at: 15th Annual Cancer Research Symposium, October 28, 2010, UC Davis Cancer Center, Sacramento, CA.
2. Invited presentation, seminar entitled: "*Integrative genomics approaches to understanding prostate cancer progression*" November 12, 2010, National Tsing Hua University, Hsinchu, Taiwan, R.O.C.
3. Tepper, C.G., Xiang, N., Ali, H.M., Gregg, J.P., Yang, J.K., Boucher, D.L, Wee, C.B., Webb, J.M., Liu, S.Y., de Vere White, R.W., Gregg, J.P., and Kung, H.J. Molecular Targeting of Prostate Cancer during Androgen Ablation: Inhibition of *CHES1*/*FOXN3*. Poster presented at: Innovative Minds in Prostate Cancer Today (IMPaCT) 2011 Conference, March 9-12, 2011, Hilton Orlando, Orlando, FL.
4. Xiang, N., Purnell, S.M., Wee, C.B., Boucher, D.L., Shi, X.B., de Vere White, R.W., Gregg, J.P., Kung, H.J., and Tepper, C.G. Amino acid-mediated mTORC1 activation is a central integration point for androgen receptor and survival signaling in prostate cancer. Poster presented at: Keystone Symposium on "*Cancer and Metabolism*", February 12-17, 2012, Fairmont Banff Springs, Banff, Alberta, Canada.

Development of expression vectors and cell lines

During the research period for this grant, we have generated a number of expression constructs and LNCaP sublines. These are listed below:

1. *CHES1* RNA interference (RNAi, Ri) expression constructs:
   A. Constitutively expressed shRNA vectors: pSM2-*CHES1*-Ri-1, -2, and -3 **(Fig. 9, 2010 Annual Report)**.
   B. Tetracycline/doxycycline-inducible shRNA expression constructs:
      1) pTRIPZ-CHES1-Ri-1, -3, and -5 **(Fig. 11, 2010 Annual Report)**.
      2) Tetracycline/doxycycline-inducible *CHES1* expression construct: pRev-TRE-HA-*CHES1*.
      3) Constitutive *CHES1*/*FOXN3* expression construct with FLAG-epitope tag: pcDNA3.1-FLAG-*CHES1.*
2. Stable LNCaP and CWR22Pc cell lines with constitutive expression of *CHES1* shRNAs:
   A. LNCaP-CHES1-Ri-1 through -9 (**Fig. 9, 2010 Annual Report)** and LNCaP-pSM2 vector control.
   B. CWR22Pc-CHES1-Ri-1/3/7 and CWR22Pc-pSM2 vector control.
3. LNCaP cells with Tet/Dox-inducible *CHES1*-targeting shRNA expression: LNCaP-tet-CHES1-Ri-1, -3, and -5 **(Fig. 11, 2010 Annual Report)** and LNCaP-pTRIPZ empty vector control.
4. *CHES1*/*FOXN3* expression constructs:
   A. Tet/Dox-inducible *CHES1* expression construct: pLVX-Tight-Puro-FLAG-*CHES1*.
5. Stable LNCaP sublines with Tet/Dox-inducible *CHES1*/*FOXN3* expression (LNCaP-*tet*-FLAG-CHES1) and corresponding vector-control cell line, LNCaP-*tet*-pLVX.
6. Stable LNCaP and 22R*v1* cell lines with constitutive expression of epitope-tagged mTOR, raptor, RagB-wt, RagB-GDP, RagB-GTP, RagD-wt, RagD-GDP, RagD-GTP.
7. Luciferase reporter constructs:
   A. *CHES1* Firefly luciferase reporter construct: pGL3B-*CHES1*-RR1/3.5
   B. *BNIP3* Firefly luciferase reporter construct: pGL3B-*BNIP3*(4.0)

8. Double-stable LNCaP and CWR22Pc sublines infected with inducible *CHES1* shRNA expression (CHES1-Ri) and *BNIP3* reporter constructs: LNCaP- and CWR22-*tet*-CHES1-Ri/BNIP3-*Rluc*. **(Task 19)**

Funding applied for based on work supported by this award

1. Cancer Center Support Grant P30 (PI: de Vere White), NCI grant 2 P30 CA93373. The *CHES1*/*FOXN3* research supported by this grant contributed greatly to the Prostate Cancer Research Program component of the P30 renewal.
2. NIH Research Project Grant (R01) (NCI) CHES1/FOXN3 function and regulation in cancer progression. Role: PI Proposed submission in October, 2013.

Employment opportunities

Thankfully, the funding from this proposal provided employment and training for several individuals, in addition to myself. Dr. Nong Xiang was an extremely talented and tremendously industrious postdoctoral fellow/Associate Research Specialist and was hired specifically for this project. She came to the UC Davis Comprehensive Cancer Center from the University of Wisconsin-Madison where she had close to seven years of experience in the prostate cancer field, which was accompanied by a significant record of productivity. She made great strides and was critical in driving this project forward. Mr. Hassen Ali (B.S. in Nutritional Biochemistry, UC Davis) was a valued research associate in my laboratory for the first 20 months of the award (April 15, 2009 – December 31, 2010) and subsequently left to pursue graduate education. Beginning in January, 2011, this position was filled by Mr. Shawn M. Purnell who had recently graduated from UC Davis with a B.S. in Neuroscience, Physiology, and Behavior (NPB). Although Hassen was a key member of the team, we were fortunate to not have any interruptions in our experiments since Shawn had already been working in the lab as a volunteer for close to one year and had been fully trained in the necessary cellular, molecular, and biochemical techniques. He provided key support for maintaining cell lines, plasmid propagation, and working with Dr. Xiang and myself to complete experiments. Due to his high level of enthusiasm and initiative, he subsequently progressed to conducting experiments almost independently. Shawn was than accepted to the University of Utah School of Medicine and left the lab in July, 2013. For the remainder of the award period, his position was filled by Ms. Tamlyn Tsubota, a recent graduate form Humboldt State University (2012) with a degree in Cellular/Molecular Biology.

Educational outreach

The research supported by this award also provided research opportunities and training to undergraduate students attending local community colleges, particularly American River College (ARC) in Sacramento, who generally cannot get this experience at their school. In conjunction with the American Medical Student Association (AMSA), I coordinated research internships for the newly developed AMSA ARC/UC Davis Cancer Center Community College Research Program." One of the interns from this program, Ms. Shanon Astey (UC Davis, 2013) worked in my laboratory on the *CHES1*/*FOXN3* project and was very helpful in preparing plasmids and performing immunoblot analyses.

CONCLUSION

The work during the research period of this award yielded several exciting and important findings that have contributed to both defining the fine mechanistic details of CHES1/FOXN3 action and a broader understanding of its global effects upon prostate cancer

progression and persistence. Taken together, our results demonstrate that *CHES1* (*Checkpoint Suppressor 1*) is a pivotal mediator of the response to androgen ablation in that is embedded in a signaling network that responds to diminished AR signaling and engenders a strong survival response through combined actions on kinase signaling pathways and transcriptional regulation of genes. As discussed previously, our proposal was based upon preliminary findings that demonstrated that *1)* that *CHES1* was an AW-induced gene that mediated PCa survival by potentially functioning as an anti-apoptotic transcription factor, particularly via its repression of the pro-apoptotic gene *BNIP3* and *2)* that heightened CHES1 expression was associated with key features of androgen ablation, specifically hyperactivation of the PI3K-Akt pathway and AR down-regulation. Our findings revealed that CHES1 is a pivotal mediator of these events by acting to further suppress AR transcriptional activity, which in turn leads to mTORC1 inactivation and a consequential de-repression of PI3K-Akt signaling. Moreover, we demonstrated that while mTORC1 responds to multiple cues, androgen and CHES1 specifically regulate its activation by amino acid signaling and at the level of the lysosome. At the same time, we fully elucidated its mechanism of action as a transcriptional regulator by demonstrating that it recruits a transcriptional co-repressor complex to the *BNIP3* enhancer/promoter and has global effects in mediating survival via its direct binding to and regulation of a large cohort of genes functioning in the negative regulation of apoptosis. Consistent with this, we find that down-regulation of *CHES1* in the context of androgen ablation effectively mediates apoptosis. In addition, *CHES1* down-regulation is required for chemotherapy-induced apoptosis and is mediated by p53-mediated transcriptional repression. Taken together, our findings provide strong support for exploiting CHES1 as a therapeutic target in that CHES1 antagonism would potentially lead to decreased anti-apoptotic PI3K-Akt signaling, combined reinstatement of pro-apoptotic gene expression (*i.e.*, *BNIP3*) and suppression of anti-apoptotic genes, and reduced activity of oncogenic, ligand-independent AR splice variants.

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SUPPORTING DATA

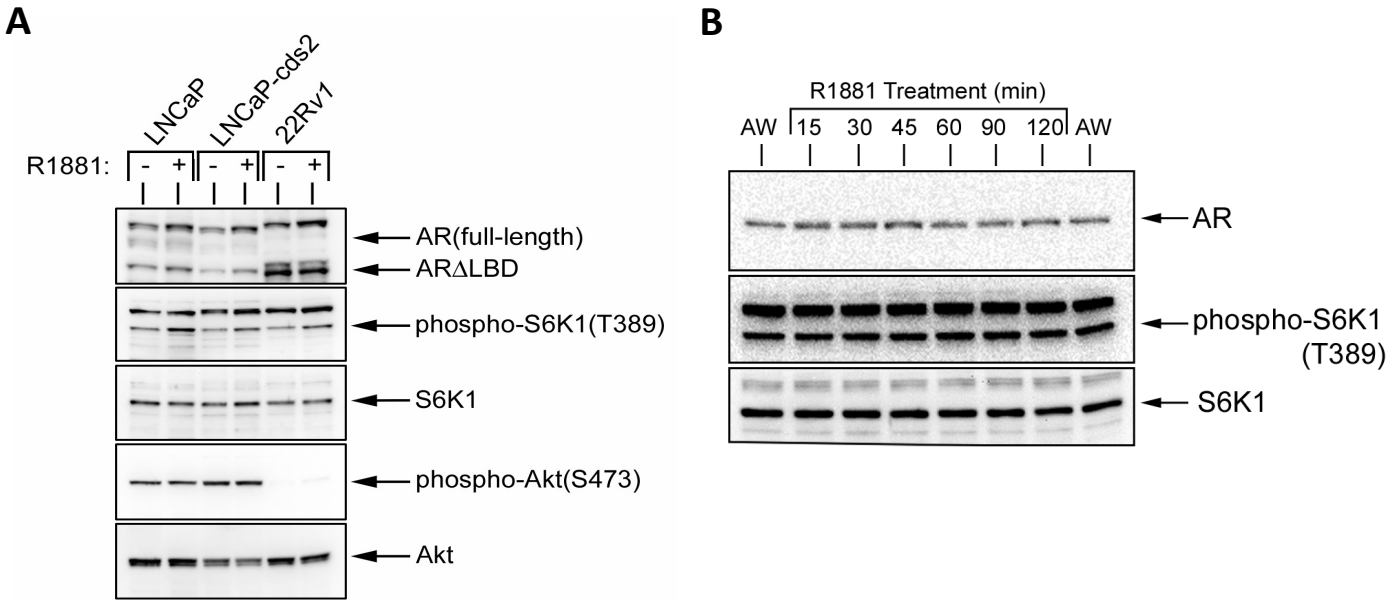


Figure 1. Androgen-responsive mTOR activation is conserved In AR-expressing androgen-independent cell lines. ***A)*** mTOR responsiveness to androgenic stimulation was examined in AR-positive and –negative androgen-independent models. LNCaP-cds2 and 22Rv1 cells exhibited androgen-inducibility of mTOR, but not of the same magnitude as that observed in LNCaP cells. ***B)*** mTOR was minimally responsive to androgen in PC3(AR)2 cells, a subline of PC3 stably transfected with an episomal AR expression construct (Heisler, L.E. et al. Mol. Cell. Endocrinol. 126:59, 1997).

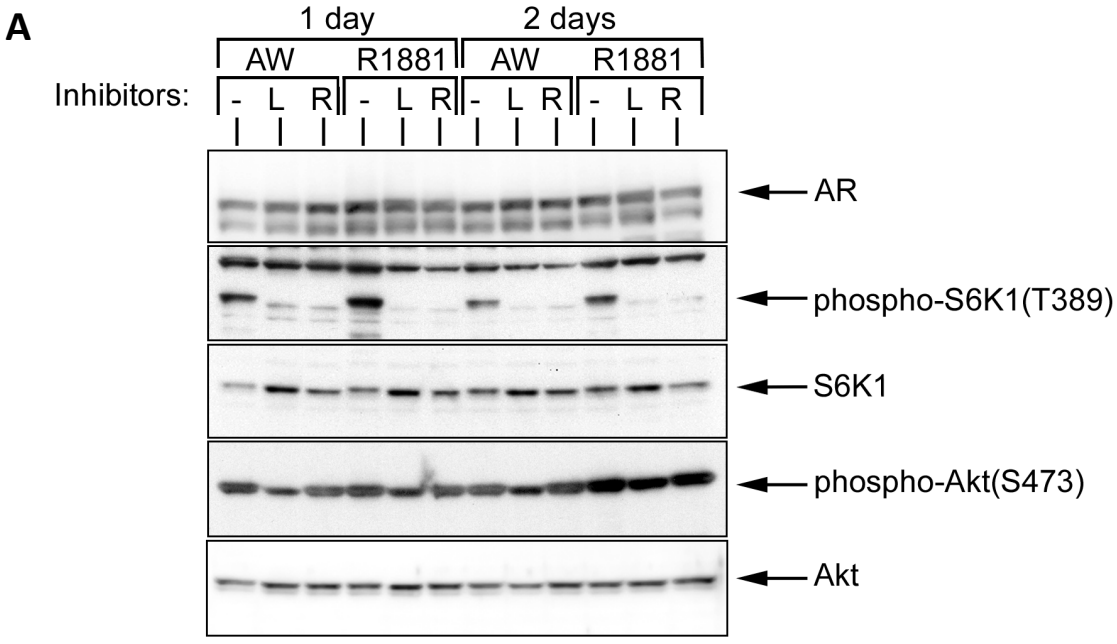
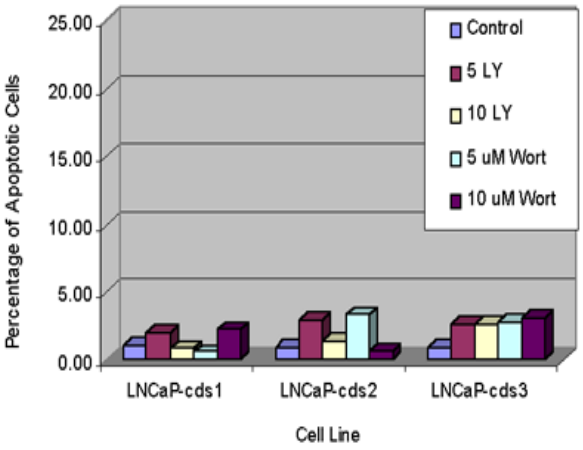
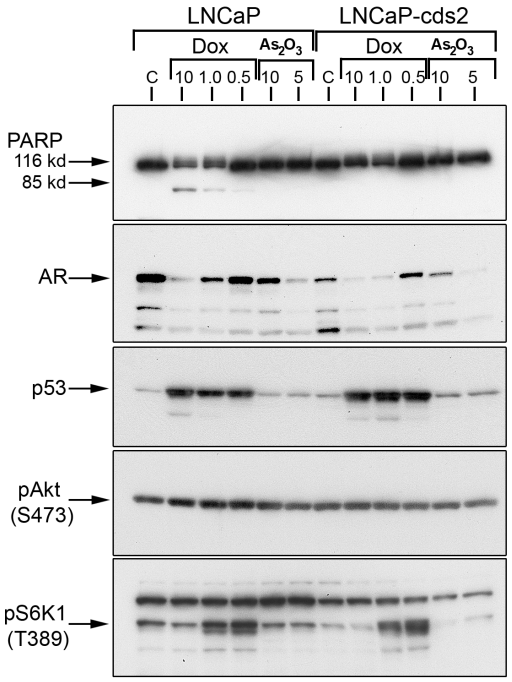


Figure 2. PI3K-Akt signaling Is dissociated from negative regulation by mTOR in androgen-independent LNCaP-cds cells. LNCaP-cds cell lines are characterized by having greatly elevated levels of AR expression and phospho-Akt(S473) compared to the parental LNCaP. Like LNCaP, R1881 treatment increased T389 phosphorylation of S6K1. In marked contrast to LNCaP, Akt was not negatively-regulated by mTOR activation. Inhibition of mTOR with rapamycin did not induce higher levels of Akt activation.





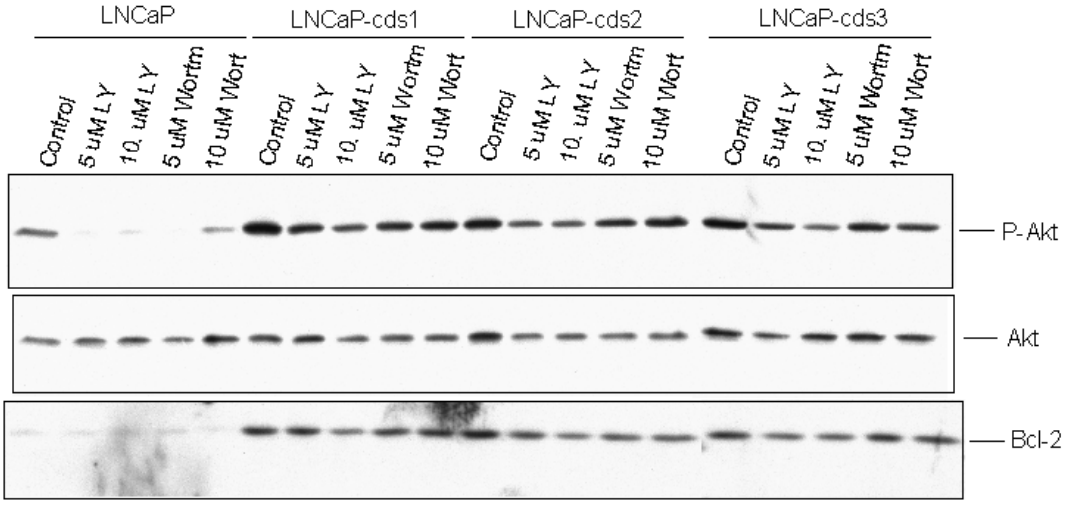


Figure 3. LNCaP-cds sublines exhibit pan-resistance to diverse apoptotic stimuli. Constitutive hyperactivation Akt and overexpression of Bcl-2 provide a molecular basis for the apoptosis-resistant phenotype of androgen-independent LNCaP sublines (LNCaP-cds). Immunoblot analyses demonstrated markedly elevated phospho-Akt levels in all LNCaP-cds clones compared to the parental line. Inhibition of PI3K abrogated Akt activation in parental LNCaP cells, but only reduced phospho-Akt levels by 50% in the cds clones. However, this level was still higher than that in the parental line. Consistent with a mechanistic link between Akt and Bcl-2, expression of Bcl-2 displayed a similar trend. The molecular aberrations in LNCaP-cds cells were manifested as a marked resistance to apoptosis induced by treatment with PI3K inhibitors. The Annexin V-FITC binding assay was used for detection and quantitation of apoptosis. The percentage of cells undergoing apoptosis after 3 days of treatment was always less than 5%. Similarly to the specific inhibition of PI3K, LNCaP-cds cells exhibited marked resistance to apoptosis induced by doxorubicin (Dox), arsenic trioxide (As_2O_3). This was indicated by the absence of detectabe PARP cleavage (*left panel*).

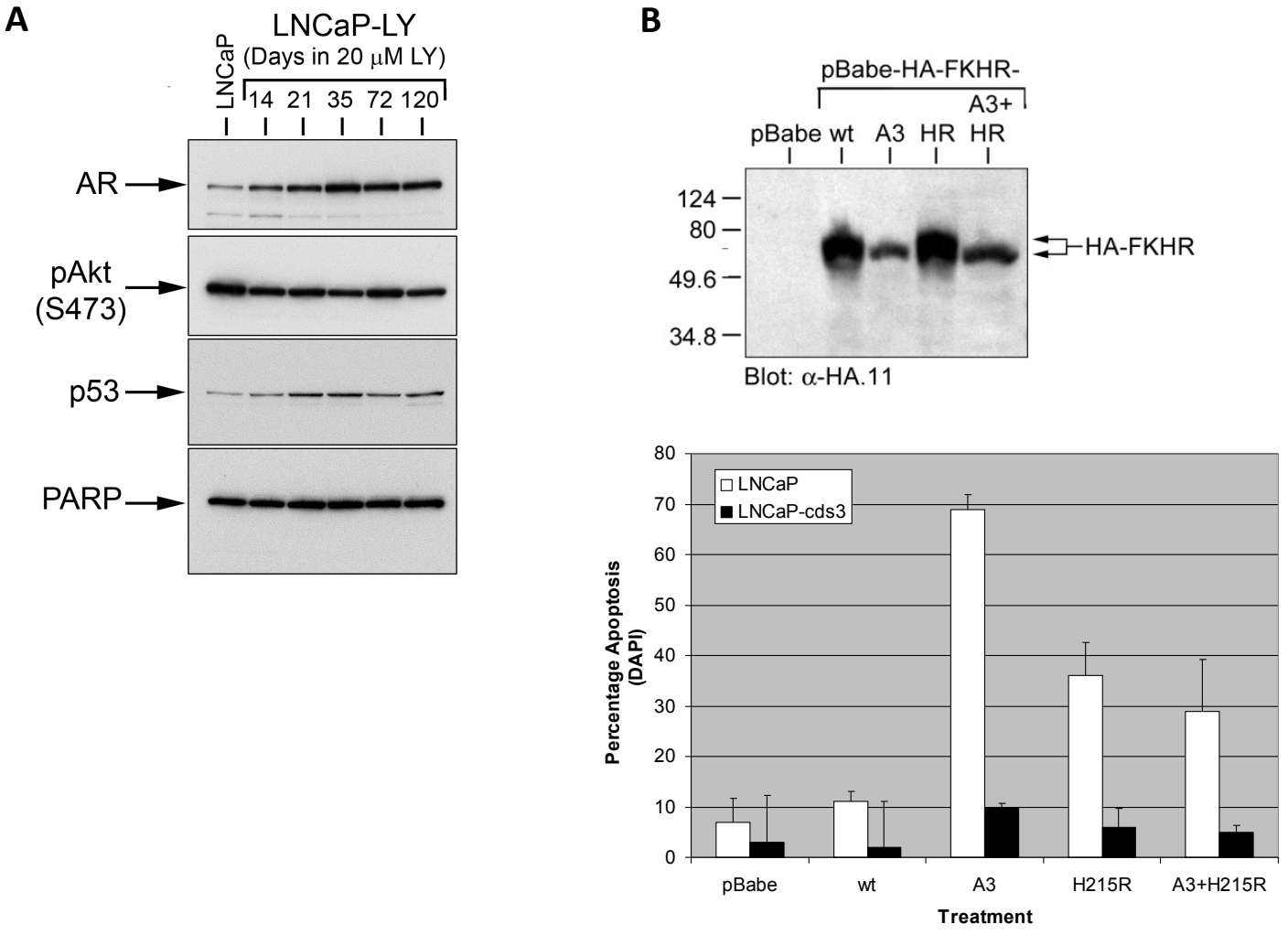


Figure 4. Aberrant Akt and downstream anti-apoptotic signaling is associated with the transition to androgen independence. ***A)*** CaP cells refractory to PI3K inhibitors exhibit features of androgen-independent cell lines. LY294002-refractory LNCaP (LNCaP-LY) cells were established by continuous culture in the presence of LY294002 (10 mM). Cultures were harvested for immunoblot analysis 14-120 days after the commencement of treatment. Similarly to LNCaP-cds cell lines, LNCaP-LY cells exhibited AR overexpression and only partial down-regulation of Akt phosphorylation. ***B)*** PI3K/Akt-independent mechanism of apoptosis resistance. In attempt to induce apoptosis in LNCaP-cds cells, PI3K-Akt signaling was bypassed by the expression of an constitutively-active FKHR mutant rendered by mutation of all three of its Akt phosphorylation sites (FKHR-A3). As demonstrated above, FKHR-A3 expression induced apoptosis of parental LNCaP, but not AI LNCaP-cds3. These results suggest that an additional anti-apoptotic mechanism is acting downstream from PI3K/-Akt to mediate resistance of LNCaP-cds cells.

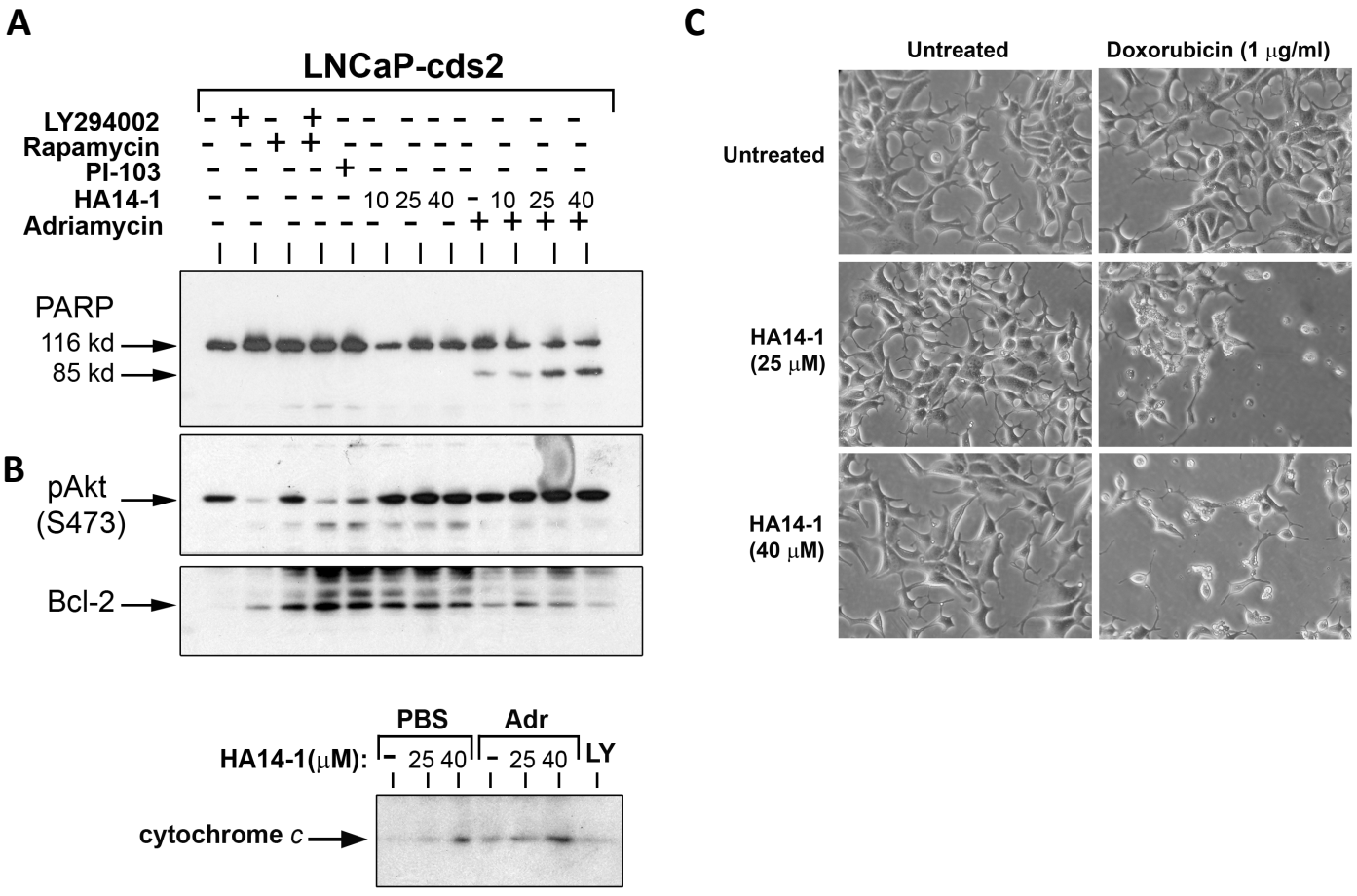


Figure 5. Inhibition of Bcl-2 function can sensitize AI LNCaP-cds cell lines to doxorubicin-mediated apoptosis. ***A)*** Based upon our previous results, we hypothesized that Bcl-2 overexpression was responsible for blocking apoptosis in LNCaP-cds3 cells in response to multiple stimuli. This was addressed by determining if these cells could be re-sensitized to apoptosis by treatment with an inhibitor of Bcl-2 function. To this end we treated LNCaP-cds2 cells with doxorubicin/adriamycin alone and in combination with HA14-1 (10, 25, 40 mM). While HA14-1 did not induce apoptosis as a single-agent therapy, Adr + HA14-1 combination treatments induced apoptosis in an HA14-1 dose-dependent manner, as demonstrated by increasing generation of the 85-kd PARP cleavage product. ***B)*** Phase-contrast micrographs of cultures treated with the indicated agents.

APPENDICES

1. Manuscript:
   Mudryj, M., and Tepper, C.G. (2013). On the origins of the androgen receptor low molecular weight species. Hormones and Cancer, 4(5): 259-269.
2. Abstract:
   Xiang, N., Ali, H.M., Davis, R.R., Liu, S.Y., Boucher, D.L., Gregg, J.P., Kung, H.J., and Tepper, C.G. Potential role of CHES1/FOXN3 as an anti-apoptotic regulator of prostate cancer response to androgen ablative and genotoxic therapies. Poster presented at: 15th Annual Cancer Research Symposium, October 28, 2010, UC Davis Cancer Center, Sacramento, CA.
3. Abstract:
   Tepper, C.G., Xiang, N., Ali, H.M., Gregg, J.P., Yang, J.K., Boucher, D.L, Wee, C.B., Webb, J.M., Liu, S.Y., de Vere White, R.W., Gregg, J.P., and Kung, H.J. Molecular Targeting of Prostate Cancer during Androgen Ablation: Inhibition of CHES1/FOXN3. Poster presented at: Innovative Minds in Prostate Cancer Today (IMPaCT) 2011 Conference, March 9-12, 2011, Hilton Orlando, Orlando, FL.
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   Xiang, N., Purnell, S.M., Wee, C.B., Boucher, D.L., Shi, X.B., de Vere White, R.W., Gregg, J.P., Kung, H.J., and Tepper, C.G. Amino acid-mediated mTORC1 activation is a central integration point for androgen receptor and survival signaling in prostate cancer. Poster presented at: Keystone Symposium on "Cancer and Metabolism", February 12-17, 2012, Fairmont Banff Springs, Banff, Alberta, Canada.
5. Manuscript in Preparation: (2011)
   Identification of *checkpoint suppressor 1* (*CHES1*)/*FOXN3* as an androgen withdrawal-induced gene mediating neuroendocrine differentiation and survival of prostate cancer cells. N. Xiang, J. K. Yang, J. M. Webb, C. B. Wee, D. L. Boucher, S. Y. Liu, C. A. Baron, R. W. de Vere White, J. P. Gregg, H. J. Kung, and C. G. Tepper
6. Manuscript in Preparation
   Xiang, N., Wee, C.B., Boucher, D.L., Ali, H.M., Liu, S.Y., Albert, S., Shi, X.B., Gregg, J.P., de Vere White, R.W., Kung, H.J., and Tepper, C.G. mTOR Integrates androgen receptor and PI3K-Akt signaling during androgen withdrawal.

On the origins of the androgen receptor low molecular weight species

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Running Head: Origins of the AR LMW species

Abstract

Prostate cancer (CaP), a commonly diagnosed malignancy, is readily treated by androgen ablation. This treatment temporarily halts the disease, but castrate resistant neoplasms that are refractory to current therapies emerge. There are multiple mechanisms by which CaP cells circumvent androgen ablation therapies, but this review focuses on the emergence of low molecular weight androgen receptor species that are missing the ligand binding domain and function independently of ligand to drive proliferation. The etiology, biological activity, unique feature, predictive value and therapeutic implication of these androgen receptor isoforms are discussed in depth.

Introduction

CaP remains one of most commonly diagnosed malignancies in the developed world[1]. Normal prostate tissue is dependent on the presence of the androgen receptor (AR) which is essential for mediating androgen signaling[2]. The AR is a steroid hormone receptor that contains 4 distinct domains: An N-terminal activation domain, a DNA binding domain, a hinge region and a C-terminal ligand binding domain[3]. The activation of AR signaling is dependent on the binding of ligand (testosterone or dihydrotestosterone), which initiates a series of conformational changes that culminate with the binding of AR to distinct DNA binding sites and an alteration of gene transcription. The dependence on AR for survival and proliferation is retained by most CaPs, hence commonly utilized treatment strategies exploit this feature by targeting the androgen receptor either with therapies that limit the levels of ligand, or with therapeutics that bind the AR to inhibit its activity[4]. These treatments provide a temporary reprieve, since castrate resistant neoplasms inevitably arise. The majority of the castration- resistant cancers continue to express and remain reliant on the AR, which appears to function despite the castrate levels of androgen[5-7]. There are multiple mechanisms by which the AR activity becomes independent of ligand binding[8], but this review will focus on the increased expression of low molecular weight forms (LMW) of the AR which are missing the ligand binding domain hence can function in the absence of ligand to ensure survival and proliferation of prostate tumor cells.

Discovery of the Truncated Forms in CWR22

Truncated isoforms of the androgen receptor (AR) in the context of prostate cancer (PCa) were originally described in castration-resistant CWR22R xenograft tumors and derivative cell lines CWR-R1[9] and 22R*v* [10]. The parental CWR22 xenograft was established from a localized tumor and has been extremely valuable for modeling prostate cancer since it faithfully recapitulates the clinical scenario in that its growth in nude athymic mice is highly androgen-sensitive, tumors regress upon castration (*i.e.*, bilateral orchiectomy), and then relapsed variants (designated CWR22R) occur 2-7 months later[11-13]). Importantly, fundamental features of androgen signaling are conserved including the expression of functional AR and secretion of prostate-specific antigen (PSA). In addition to being activated by testosterone and 5α-dihydrotestosterone, the CWR22 AR possesses an H874Y mutation resulting in broadened ligand specificity for estradiol and progesterone, elevated responsiveness to the adrenal androgen dehydroepiandrosterone (DHEA), and stimulation by the antiandrogen hydroxyflutamide[14]. The findings of truncated ARs in 22R*v1* were particularly exciting since antibody mapping implicated the 75-80-kD truncated AR species as being constitutively active due to their lack of the regulatory ligand-binding domain (LBD), and thereby represented a novel mechanism for evading androgen deprivation [10]. The functionality of the ARΔLBD was validated by its ability to localize to the nucleus, bind to AR consensus DNA binding sites (*e.g.*, ARE, ARR) with high affinity in the absence of androgen, and activate *PSA*/*KLK3* and *probasin* promoter-driven reporter constructs under conditions of

androgen deprivation. Interestingly, there were two, easily-discernible forms of the ARΔLBD, which became more prominent under these conditions. Another noteworthy feature of the 22R*v1* AR repertoire was the expression of an extended full-length AR (114 kD) that was approximately 4 kD greater in mass than LNCaP AR protein. This was derived from the insertion of an additional 39 amino acids derived from an in-frame tandem duplication of exon 3 (117 bp) that encodes the second zinc finger of the DNA-binding domain (DBD) [10], which resulted from an 35-kb genomic rearrangement around this locus [15] Screening of a small panel of CWR22/CWR22R tumors demonstrated that the exon 3 duplication mutation (AR-E3DM) originated in the CWR22R-2152 relapsed tumor from which 22R*v1* was derived, which notably was the most aggressive relapse [13]. It was also demonstrated that truncated AR proteins could be derived from the full-length AR by proteolytic processing and that the AR-E3DM mutation sensitized the AR to this process. Although the AR had been demonstrated to be a suitable substrate for caspases [16] and ubiquitin-proteasome processing [17], the treatment of 22R*v1* cells with their respective inhibitors did not diminish the levels of truncated AR. In contrast, it was later shown that calpain-mediated AR cleavage was a prominent mechanism leading to generation of the truncated AR [18]. Since the initial discovery of AR truncated isoforms, there have been intense efforts directed towards characterizing their composition, better defining their functions, elucidating the mechanisms leading to their generation, and therapeutic targeting. These will be discussed below. Following the discovery of the LMW-AR forms different groups proposed various hypotheses on the etiology of these AR isoforms.

Post-translation processing of full-length AR into truncated isoforms

Calpains, calcium-dependent proteinases, are ubiquitously expressed and proteolyse numerous substrates. In general, calpains cleave proteins at a limited number of sites to generate large polypeptides[19], and proteolysis can serve to alter protein activity or localization. Several members of the steroid hormone receptor superfamily, including the estrogen[20] and glucocorticoid[21] receptors are calpain substrates. Biochemical analyses demonstrated that the AR can be proteolyzed by calpain to fragments ranging from ~75 kDa to 34/31 kDa[22] , with the the 75-kDa fragment having retained the N-terminal domain thereby suggesting that proteolysis removed the C-terminal region of the molecule (Figure 1). Follow-up studies demonstrated that *in vivo* stimulation of calpain activity in 22R*v1* cells resulted in a decrease in full length AR (FL-AR) but not of the LMW-AR[18]. Conversely, treatment of the cells with a calpain inhibitor moderately decreased the level of the LMW-AR. Since calpain cleavage is dependent on protein structure and not simply sequence[23] the insertion of an additional third exon, as found in the 22R*v1* AR, would alter AR structure and may sensitize the mutant molecule to proteolysis. CWR-R1 cells express higher levels of calpain 2 than 22R*v1* cells and higher levels of ERK[24], a kinase that phosphorylates and activates calpain 2[25]. The expression of LMW-AR expression was reduced by treatment with the calpain inhibitor calpeptin and an siRNA targeting calpain 2 or by inhibition of ERK activity. Additionally, expression of the FL-AR cDNA in PC3 cells, which express only trace levels of AR, resulted in the expression of the FL-AR as well the as ~80 kD ARΔLBD forms[24]. Subsequent studies reported that treatment of LNCaP cells, which do not express detectable levels of the ARΔLBD, with the proteasome inhibitor bortezomib resulted in the generation of a C-terminally truncated ARΔLBD [26]. The authors propose that treatment with bortezomib leads to the activation of calpain and calpain-dependent proteolysis of the AR. Studies by Harada[27] found that in a castrate-resistant LNCaP derivative the low levels of the C-terminally truncated AR were enhanced by androgen deprivation, and treatment with a proteasome inhibitor in conjunction with bicalutimide greatly augmented the generation of the truncated AR. It is notable that calpain 2 levels are low in benign prostatic hypertrophy, are elevated in localized prostate cancers, and are highest in metastatic

lesions[28]. In total, these studies support the notion that proteolysis of the FL-AR is one mechanism that results in the expression of the castrate-resistant ARΔLBD forms and contributes to the development of castrate resistance.

Premature chain termination generates a C-terminal deleted AR

Studies of AR mutations in castrate-resistant metastatic cancers led to the identification of a mutation at amino acid position 640 (Q640Stop), which resulted in premature chain termination and the generation of a truncated AR[29]. This mutation mapped to the AR hinge region giving rise to a protein that include sequences encoded by exons 1, 2, 3 and part of 4 (Figure 1A). The identical mutation was later identified in a different metastatic prostate cancer[30]. The same study identified additional signal basepair mutations, which would result in premature chain termination and the generation of a truncated AR, indicating that such mutations are not rare events. The Q460Stop mutant protein requires phosphorylation[31], could homodimerize[32], and could transactivate expression of certain genes in castrate levels of androgen. However, AR-Q640Stop-FL-AR heterdimers were required for activation of additional AR targets under androgen deprivation conditions[32]. An analysis of the interaction of the AR-Q640Stop mutant revealed that CBP and c-Jun were highly recruited by the mutant AR leading to an activation of AP-1, NFAT and NF-κB transcriptional activity. This property was not observed with the FL-AR, suggesting that it is restricted to the Q640Stop mutant and may be a mechanism by which it contributes to castrate resistance[31]. Consistent with the finding that Q640Stop phosphorylation is required for activity, the multikinase inhibitor sorafenib inhibited FL-AR and Q640Stop activity in castrate resistant cells[33].

Truncated AR variants resulting from alternative splicing

An AR splicing variant in a patient with androgen insensitivity syndrome (AIS) was reported over two decades ago[34]. Shortly afterwards, a study on breast cancers identified an identical AR splice variant that was expressed in a number of breast tumors, but not in normal breast tissue[35]. In both cases the splice variant had a deletion of exon 3, and encoded a protein that was missing the second zinc finger of the DNA binding domain (Figure 1A). The authors of the breast cancer study suggested that given the reduced capacity of such a deletion to bind DNA, the presence of the splice variant in breast tumors would serve to decrease the growth-inhibitory role of the AR in breast tumors and therefore contribute to tumorigenesis. The identification of an identical mutation in AIS strongly supports the hypothesis that the second zinc finger is important for AR function. There are no subsequent follow-up studies on AR splice variants in breast cancer, but several years ago a number of studies detected AR splice variants in prostate tumor-derived cells. The first documented expression of a splice variant in metastatic prostate cancer resulted in an insertion of an intron 2 sequence that encoded 23 amino acids between the two zinc fingers of the DNA binding domain[36]. This splice variant was shown to localize predominantly to the cytoplasm, but in LNCaP cells could translocate into the nucleus and exhibited partial transcriptional activity. In this case, the insertion of additional sequences between the two zinc fingers compromised AR function.

The majority of studies on AR splice variants utilized the 22R*v1* cell line. This line expresses abundant levels of ARΔLBD forms that differ somewhat in molecular weight. One study reported the expression of a truncated AR form, which was encoded by an mRNA that contained a novel exon (exon 2b) at the 3'-end[37]. This splice variant contained the N-terminal transactivation domain, the first zinc finger and the novel exon 2b derived sequence. An siRNA targeting the novel exon was able to reduce the expression of the low molecular weight AR forms. The 1, 2, 2b splice variant could also be detected

in VCaP and LAPC4 cells, although at low levels, as well as in two xenograft cell lines of prostate cancer progression- LuCaP23.1 and LuCaP53.

Additional studies reported that the 22R*v1* cell line expressed a number of splice variants. Several of the variants reported by two groups were identical, and several contained the duplicated exon 3 sequence, hence were specific to the 22R*v1* cell line[38, 39]. However, the majority of variants had only one exon 3 but novel sequences derived from different regions of intron 3 [40, 41]. One particularly abundant transcript, designated as either AR3[38] or AR-V7[39] (henceforth referred to as AR3/AR-V7), consisted of exons 1, 2, 3 and a novel sequence derived from intron 3 which resulted in the addition of 16 unique amino acids at the C-terminus of the protein. The other unique C-terminal extensions derived from various regions of intron 3 differed in sequence and in size. Some novel transcripts contained novel intron 2 derived sequences, but these were less plentiful. These studies indicate that there are two hotspots in the AR gene that give rise to alternative splice variants –intron 2 and intron 3, with the latter being the most common.

Studies utilizing a yeast functional assay led to the identification of two novel transcripts that contained an insertion between sequences encoded by exons 3 and 4 derived from an intron[42]. Since the insertion resulted in a premature termination codon, the transcripts, while containing sequences corresponding to all of the exons, encoded a protein that contained the N-terminal, the DNA binding domains, as well as unique amino acids derived from the inserted intron 3 sequence. Hence the repertoire of AR splicing variants expressed in 22R*v1* cells further increased. The protein product generated therefore resembled the previously identified variants in that it consisted of the N-terminal domain, the two zinc fingers, and a novel sequence encoded by intron 3.

Analyses of AR transcripts in two castrate resistant LuCaP xenografts led to the identification of a novel splice variant ARv567es that contained sequences for exons 1, 2, 3 and 4[43]. The splicing of exon 4 to exon 8 resulted in a frameshift that generated a stop codon after the first 29 nucleotides, thus the exon 8 sequence encoded a novel 10-amino acid sequence at the C-terminus of the variant protein (Figure 2B). Very similar transcripts were detected in independent studies using castrate resistant tumor tissue[44]. However, these transcripts differed in their 3' untranslated region where a portion of exon 8 was replaced by sequences mapping 3' of the gene, which the authors named exon 9. While these transcripts were abundant in castrate resistant tumor tissue, they were less abundant in 22R*v1* cells. These results add an additional layer of complexity and argue that expression of specific splice variants is in part dependent on cellular context.

Biology of AR variants: cellular biology, activity, localization, and expression in malignancies

The splice variant consisting of exons 1, 2 and 2b was able to transactivate transcription in an androgen-independent manner[37]. While there is no evidence that this splice variant can translocate into the nucleus, its ability to transactivate transcription strongly suggests that it is able to enter the nucleus. In the LuCaP xenograft model the expression of this splice variant in cells that acquired castration resistance was higher than in the castrate sensitive parental lines, indicating that androgen deprivation promotes expression of this splice variant[37].

The AR3/AR-V7 has been particularly well analyzed. Studies revealed that the transcript is expressed in CWR-R1, VCaP cells, and in hormone-refractory prostate tumors. AR3/AR-V can promote transcription in an androgen-independent manner and can function in the absence of FL-AR. Ectopic expression of AR3/AR-V in castrate sensitive LNCaP cells confers castrate resistance. The expression of this variant is elevated in castrate resistant tumors when compared to hormone naïve cancers[39] and correlates with tumor progression[38]. The latter assertion has been challenged by studies which argue that transcript levels of AR-V1 and AR3/AR-V7 do not predict recurrence in patients[45].

Studies of Guo[38] and Hu[39] reported that AR3/AR-V7 localizes to the nucleus and cytoplasm in cultured cells and in tumor tissue, and nuclear localization is not dependent on the presence of the full-length AR. An additional study conducted in COS1 cells showed that the expression of AR3/AR-V7, ARv567es, as well as several other splice variants is predominantly nuclear, but can be detected in the cytoplasm[46]. Guo and colleagues also observed that antibodies generated against the unique AR3/AR-V7 sequence detected expression in prostatic basal and stomal cells, but not epithelial cells in benign tissue. However, in malignant tissue, the majority of luminal epithelial cells exhibited strong staining, and in castrate resistant tumors there was a redistribution of AR3/AR-V7 protein expression to the nucleus. The splice variant is missing the LBD and as anticipated is refractory to enzalutamide, a second-generation AR antagonist that targets this region[47]. Interestingly the activity of the AR5/AR-V7 variant has been shown to be regulated by the PTEN-AKT pathway mediated by FOXO1[48].

The ARv567es splice variant has also been the subject of several inquiries. Like AR3/AR-V7, it is constitutively active[43]. The cellular localization of this variant is predominantly nuclear in both the presence and absence of ligand. Moreover, ectopic expression of ARv567es in LNCaP cells not only conferred castration resistance, but enhanced the expression of the endogenous FL-AR, suggesting that ARv567es can autoregulate endogenous AR transcription. Notably, in this context, ARv567es was more effective at transactivating known AR target genes than the FL-AR. Additional studies suggest that ARv567es can physically interact with the FL-AR and stabilize the FL-AR protein[43].

An analysis of AR3/AR-V7 and ARv567es transcripts in normal and metastatic samples showed that approximately 30% of the metastatic lesions and 17% of normal tissue express ARv567es, while 16% of metastatic lesions and 6% of normal samples express the AR3/AR-V7 splice variant[43]. An independent study indicated that an ARv567es-like transcript identified in metastatic tumors also was elevated in metastatic lesions, when compared to hormone-naïve tumors[44].

The expression levels of ARv567es, AR3/AR-V7, and AR-V1 were assessed in bone metastases[49]. The AR-V1 and AR3/AR-V7 transcripts were detected in most non-malignant tissue, primary tumors, and metastases, and levels were elevated in metastatic lesions. In contrast, the ARv567es variant was detected in 23% of the metastases but not in primary tumors. The differences between the above-mentioned study and this analysis may be due to an analysis of different types of metastatic lesions or differences in interpretation and assigning cutoffs for expression at distinct levels. The study of bone metastases also analyzed AR protein expression in a small cohort of tumors using Western immunoblots. Metastatic tumors with the highest levels of AR3/AR-V7 had the highest levels of the ARΔLBD form, while tumors with the lowest level of AR3/AR-V7 had the lowest ARΔLBD levels. However, the levels of ARv567es mRNA did not correlate with levels of the ARΔLBD protein. The Western blot analysis showed that the AR variant proteins constituted ~32% of the FL-AR. The authors also noted a discrepancy between protein and RNA ratio of FL-AR and AR3/AR-V7, where the RNA ratio of FL-AR and AR3/AR-V7 (0.4% of the FL-AR) was almost two orders of magnitude lower than the ratios of FL-AR and ARΔLBD protein forms. This led to the suggestion that the difference may be due to possible post-transcriptional stabilization of the splice variants. An alternative explanation is that, as in 22R*v1* cells, there are a number of low molecular weight forms of AR, and the AR3/AR-V7 splice variant encodes a fraction of the observed ARΔLBD protein. However, the correlation of high AR3/AR-V7 mRNA levels and high levels of the ARΔLBD forms suggests that this variant may be a marker of increased aberrant splicing. While elevated expression ARv567es was not indicative of levels of the ARΔLBD protein, it was associated with intense nuclear AR staining.

Some of the proteins encoded by the splice variants localized to the cytoplasm rather than the nucleus. One such variant, AR8, is missing the DNA binding domain due to the utilization of an alternative exon 3 splice acceptor and a novel intron 3 sequence. The variant is associated with the

plasma membrane[41] through palmitoylation of two cysteine residues within its unique C-terminal sequence. As with the other splice variants, the levels of AR8 are elevated in castrate-resistant cells. Overexpression of this this splice variant promoted the interaction of Src and AR with EGFR in response to EGF and subsequently increased AR tyrosine phosphorylation[41]. Thus the AR8 isoform may promote castration resistance in a nonorthodox manner; by augmenting a response to growth factor signaling.

If LMW-AR forms are associated and possibly predictive to prostate cancer progression, then defining tumors or focal regions of tumors that express these forms would be important in designing therapeutic strategies. The methodologies employed in most studies utilized RT-PCR analysis of mRNA isolated from frozen tumor tissue to detect expression levels of specific splice forms. While these studies have established that castrate-resistant tumors express elevated levels of specific variant transcripts, they are detecting AR variants in a tissue that, unless microdissected, contains a heterogeneous population of cells and only one variant transcript is analyzed in a single reaction. An alternative approach is to use Western immunoblot analysis to define the expression of the 80-kDa AR protein. This method, which would detect all LMW-AR forms, is even more problematic, since sufficient amount of frozen tissue is required and as with the mRNA analysis, the assay detects protein expression in a heterogenous mixture of cells. To circumvent these problems, Zhang and co-workers developed an immunohistochemical approach utilizing antibodies that recognize the N-terminal and C-terminal domains of the AR molecule[50]. They reasoned that the ratio of staining intensity obtained with the two antibodies was reflective of the expression levels of ARΔLBD forms. The results obtained showed an overall high frequency of C-terminal truncated AR variants in castrate-resistant tumors, leading the authors to argue that this method could be used to stratifying patients for AR targeting therapeutics.

Transcriptional signature of AR variants

Several studies sought to identify a transcriptional signature of the AR variants. Tsai *et al.* generated a recombinant LNCaP subline that inducibly expressed a 22R*v1*-derived truncated AR (TC-AR) corresponding to the Q640Stop mutant and containing the exon 3 duplication and found that overexpression of this variant greatly reduced the levels of endogenous AR transcription[51]. This contrasts with the results obtained when ARv567es was ectopically expressed in LNCaP cells[43]. The ability to induce the expression of the truncated receptor allowed for the study of Q640Stop and FL-AR properties in an isogenic background. As previously shown, the variant can translocate into the nucleus and transactivate transcription in castrate levels of androgen. The study found that there is a significant overlap in the cohort of genes regulated by FL-AR and the TC-AR, but also identified transcripts that were uniquely transactivated by the Q640Stop, including *RHOB*, which encodes a protein involved in migration and the morphological changes that are observed when Q640Stop is overexpressed in LNCaP cells.

An siRNA-mediated decrease of AR3/AR-V7 expression in 22R*v1* cells cultured in castrate androgen levels identified a set of transcripts that were specifically regulated by AR3/AR-V7, but not FL-AR. One AR3/AR-V7-regulated transcript AKT1, a serine threonine kinase was further analyzed. Expression of this transcript was also decreased in CWR-R1 cells following AR3/AR-V7 ablation and regulated AKT1 expression in this cellular context as well. Moreover, AKT1 ablation in CWR-R1 cells reduced cellular proliferation.

A similar analysis was conducted in the CWR-R1 cells using a sub-cell line that was enriched for an intron 1 deletion and had elevated levels of the AR variants. However, changes in AKT1 expression were not observed following ablation of the AR3/AR-V7 variant. Additional gene profiling studies indicated that many, but not all genes were regulated similarly by ligand-activated FL-AR and the

constitutively active splice variant. The authors argue that this result explains why androgen and the ligand-independent variants can interchangeably promote growth of the same cells[47].

Ectopic expression of AR3/AR-V7 in LNCaP cells, approximating the levels observed in castrate resistant tumors identified a set of transcripts that were distinct from the cohort of genes transactivated by FL-AR. The most highly transactivated AR3/AR-V7 gene set consisted predominantly of transcripts that were associated with cell cycle regulation. In contrast, the most highly expressed FL-AR specific cohort consisted of genes that were related to biosynthesis, metabolism and secretion. The AR3/AR-V7-specific gene set was also transactivated by ectopic expression of the ARv567es variant, suggesting that both splice variants target the same genes. The expression of this cohort of transcripts was further validated in LNCaP95 cells, which express elevated levels of the AR3/AR-V7 variant. Ablation of FL-AR and AR3/AR-V7 variant, but not ablation of FL-AR alone, resulted in decreased expression of the AR3/AR-V7 specific set of transcripts, confirming that this variant is required for transcript expression. An analysis of one AR3/AR-V7 specific transcript, UBE2C, in castrate resistant cancers indicated that UBE2C expression correlated with AR3/AR-V7, but not FL-AR levels.

The gene expression program regulated by the ARv567es variant was independently assessed by ectopic expression in LNCaP cells[43]. The variant specific set of transcripts was compared to transcripts that were regulated in control LNCaP cells following androgen addition. The study revealed the variant could transactivate well known androgen-regulated transcripts in the absence of ligand as well as ARv567es-specific targets that were not affected by the addition of androgens. Subsets of variant-specific targets increased in the absence of androgen were genes whose functions were classified as transcription factor activity and genes that have roles in steroid biosynthesis and metabolism. It is notable that the ARv567es-dependent regulation of certain androgen-responsive genes was sometimes opposite from that of FL-AR-dependent regulation. The gene expression program of the multiple other splice forms has not been analyzed, so at this time it is unclear which, if any, of the unique gene targets are common to more than one splice variant.

Biological implications of splice variants

While all of the above-cited studies identified splice variant-specific transcripts, there was almost no overlap in the gene sets obtained in the different studies. This could be in part due to the different cellular backgrounds, different specific splice variants, and ectopic expression vs. siRNA-mediated ablation of variant expression. We had previously shown that the androgens directed a different transcriptional program in the two related cell lines 22R*v1* and CWR-R1, even though the AR interacted with almost identical DNA sites[52]. AR-dependent transcription is governed by a large number of co-regulators, which are likely to be differentially expressed in distinct cell lines. However, a number of studies utilized the castrate sensitive LNCaP cells where various splice variants were ectopically expressed in the presence or absence of androgens. The individual studies used somewhat different conditions and levels of variant expression, and data sets were evaluated using different criteria. These could all affect the identification of transactivation (or repression) of specific targets and since the studies further stratified on the basis of the most elevated or repressed targets, the results obtained would be expected to vary.

Different variants may well have variant-specific unique properties. The ARv567es localizes almost exclusively to the nucleus and has been shown to stabilize FL-AR levels in LNCaP cells. In contrast, the AR3/AR-V7 splice variant localizes to nuclear and cytoplasmic compartments and there is no evidence that it stabilizes the endogenous FL-AR. Furthermore, the Q640Stop variant, when overexpressed in LNCaP cells, represses the expression of the FL-AR. These distinct features could all affect the activity and variant-specific transactivation of gene transcription.

All of the variants used in these studies described above share certain features; 1) they can localize to the nucleus in the absence of androgen, and 2) they confer castrate resistance. This last property indicates that the variant can initiate a cell cycle progression program with an efficacy that is similar to the efficacy of ligand-activated FL-AR. The simplest explanation is that the same critical genes are targeted. However, the critical target genes may not be the same as the most highly transactivated or repressed genes. It is most likely that the variants that are missing the LBD and the FL-AR will have unique properties. A number of AR-interacting proteins, which modify AR activity are dependent on the presence of the LBD (ARA54, ARA55, Hsps, Tip60), hence the variants that are missing this domain would not be subject to modulation by these co-regulators. This has been validated by studies which showed that AR splice variants are resistant to Hsp90 inhibitors[53]. In contrast, Vav3 an AR co-activator which requires the AR N-terminal domain can effectively interact with and activate AR3/AR-V7 and ARv567es[54].

Genomic predisposition associated with differential splicing

The commonly used models for the study of AR variant expression 22R*v1* and CWR-R1 harbor AR genomic aberrations, leading to a hypothesis that such aberrations promote alternative splicing. 22R*v1* cells have a 35-kb duplication of exon 3 and flanking sequences. CWR-R1 subclones, which express higher levels of the ARΔLBD have a 48-kb deletion in AR intron 1. The LuCaP 86.2 xenograft, which expresses only the ARv567es variant has an 8,579 bp intragenic deletions of AR exons 5, 6 and 7[55]. Another example that supports this hypothesis is the discovery that alternatively spliced AR mRNAs were expressed in a mouse prostate tumor derived from the Myc mouse model of prostate cancer[56]. Notably, a novel sequence contained in the mRNA maps 5' of the AR gene. Since transcription does not loop back, the most plausible explanation is that in this tumor the AR has a genomic abnormality, placing the novel sequence 3' of the transcriptional start site. However, it is unclear if a genomic alteration enhances variant expression or if it is required for variant expression. Given that certain variants are expressed in benign tissue as well as in primary prostate tumors, AR genomic aberrations are either extremely common, or genomic aberrations further potentiate splice variant expression.

Regulation of alternative splicing

The information required to define RNA regions of the preRNA that will be included in the mRNA is present in the sequence, but this information is discerned by RNA binding proteins (RBP), hence the levels and activity of these proteins regulate alternative splicing. These factors interact with exonic and intronic regions of the nascent mRNA, regulating the recruitment and activity of the spliceosome[57]. Some proto-oncogenes, including cyclin D1 and H-Ras, can be alternatively spliced to yield proteins that have distinct properties[58].

Several studies have reported that the levels of AR splice forms increase when cells are placed in castrate levels of androgen. This increase occurs at the transcriptional level within days, and is coincident with a decrease in the levels of the FL-AR suggesting that there is a shift in AR mRNA splicing. *In vivo* and *in vitro* studies have indicated that castration induces oxidative stress through redox imbalance by up-regulation of ROS-producing and down-regulation of ROS-detoxifying enzymes[59]. AR levels and the levels of some AR co-regulators has been reported to be activated by oxidative stress, suggesting that reducing androgen-dependent AR signaling may have an active role in the acquisition of castrate resistance.

It is noteworthy that shifts in splicing of certain transcripts have been observed when cells are undergoing a stress response[60]. The p53 regulators MDM2 and MDM4 are alternatively spliced when

cells are exposed to irradiation [61]. Moreover, in rhabdomyosarcoma cells the alternative splicing of MDM2 in response to by UV radiation and cisplatin is dependent on MDM2 intronic elements[62]. Hypoxic stress has been shown to induce alternative splicing of presenilin 2 in human neuroblastoma cells [63], and nutrient starvation inhibits the splicing of G6PD gene transcripts in mouse liver[64].

During a stress response, various splicing factors (hnRNP A1, Slu, DDX5)[65-67] are relocalized to the cytoplasm, where they play a role in translation and stability of mRNA. The splicing factor Sam68 localizes to nuclear stress granules in response to heat shock and TOP2 inhibitors[68]. Sam68has been shown to modulate the splicing of cyclin D, and levels of Sam68 correlate with the levels of cyclin D1b, a splice variant that unlike cyclin D1a can transform cells, promote anchorage independent growth, and increase cellular invasiveness[69]. A polymorphism at a cyclin D splice site promoted the expression of the cyclin D1b variant[70]. Modification of splicing components has not been well explored in the context of prostate cancer or in the etiology of castrate resistance. If androgen deprivation modifies the availability or activity of splicing factors, then such changes may shift the AR splicing pattern in some tumors to favor the expression of the low molecular weight splice variants. The precedent that intronic regions of the MDM2 gene contribute to alternative splicing suggest a unifying hypothesis, where genomic alterations such as intronic deletions or duplication predispose the AR gene to alternative splicing that is further facilitated by a modulation of splicing factors by androgen deprivation.

Biological and Therapeutic implication

Various studies have shown that AR variants that are missing the LBD are constitutively active and can function independently of the FL-AR. Therefore these variants will be refractory to therapeutics that target the LBD. But most tumors express the FL-AR and the LMW forms, so LBD targeting therapeutics may have some efficacy is retarding tumor growth. Yet, the benefit from such therapies would be temporary since studies have shown that androgen withdrawal promotes the expression of the LMW-AR. Hence the tumors would adapt and become more dependent on the variants that are missing the LBD. Targeting the N-terminal domain of the AR would be a more prudent strategy since all the variants and the FL-AR contain this region. The NTD-targeting agent, EPI 001 has been shown to inhibit proliferation of 22R*v1* cells culture in castrate androgen levels, while the LBD-targeting agent bicalutamide failed to affect cell growth[71]. This agent has promise for treatment of tumors that are reliant on the activity of the ARΔLBD. If the ARΔLBD is generated by increased proteolysis then protease inhibitors may affect proliferation. An alternative strategy is to target critical N-terminal domain interacting proteins. Studies have shown that a disruption of the p160 co-activator interface with the androgen receptor can inhibit the activity of androgen-dependent and castrate resistant prostate tumor cells[72]. ASC-J9, an agent that promotes AR degradation, has been shown to affect FL and ARΔLBD [73], and compounds found in the Kava root have been shown to decrease the levels of FL and ARΔLBD in a xenograft model of prostate tumorigenesis[74]. Future therapies may include the targeting of variant specifically using an RNAi approach, but at this time these are in an early stage of development.

The presence of ARΔLBD forms is common and has been detected in malignant and non-malignant tissue, but the levels and perhaps the diversity of LMW-AR forms is greater in higher grade neoplasms, particularly in castrate-resistant tumors. Moreover, there are several mechanisms that can give rise to these forms and each is associated with more advanced malignancies. The association of ARΔLBD forms with cancer progression is clear, but this observation is correlative. The studies that demonstrate a causative role for the LMW-AR in the etiology of castrate resistance are reliant on studies in the few prostate tumor-derived cell lines. Nevertheless, all of the studies indicate that these forms promote castrate resistance. While some of the splice forms do not retain the DNA binding domain,

most do. The deletion of the LBD and retention of the N-terminal transactivation region and DNA binding domain, indicates that they have the potential for promoting transcription. The cell culture studies show that overexpression of the ARΔLBD forms is sufficient to promote proliferation, but in many of these studies the levels of the ARΔLBD are very high. While the discovery and characterization of truncated AR variants has advanced the field of PCa research, it also has prompted the emergence of numerous questions. So, are ARΔLBD forms the drivers of castrate resistance in tumors? In this respect, much more work needs to be done to define the predictive value of the ARΔLBD expression. Do all ARΔLBD forms correlate with castrate resistance or are specific splice variants associated with the disease? Some studies suggest the latter, but these results need to be solidified. Do even low levels of the specific LMW-AR portend a poor clinical outcome, or is there a threshold effect? Do splice variants transactivate specific target genes or are they simply able to function in castrate levels of androgen to drive cell proliferation? If a splice variant has specific targets are these responsible for, or contribute to, disease progression? Is there a splice variant signature that can act as a marker for predicting outcome? At this time a specific signature cannot be discerned but with additional data sets a pattern may emerge. Lastly, can the mechanisms that lead to the expression of ARΔLBD be targeted by therapeutics? This necessitates deciphering the molecular details of such mechanisms. Identification of AR variants that are refractory to androgen ablation therapy was a great step in defining a possible cause of castrate resistance. The next steps are to solidify these finding and to ultimately design therapies that can circumvent this roadblock to prostate cancer treatment.

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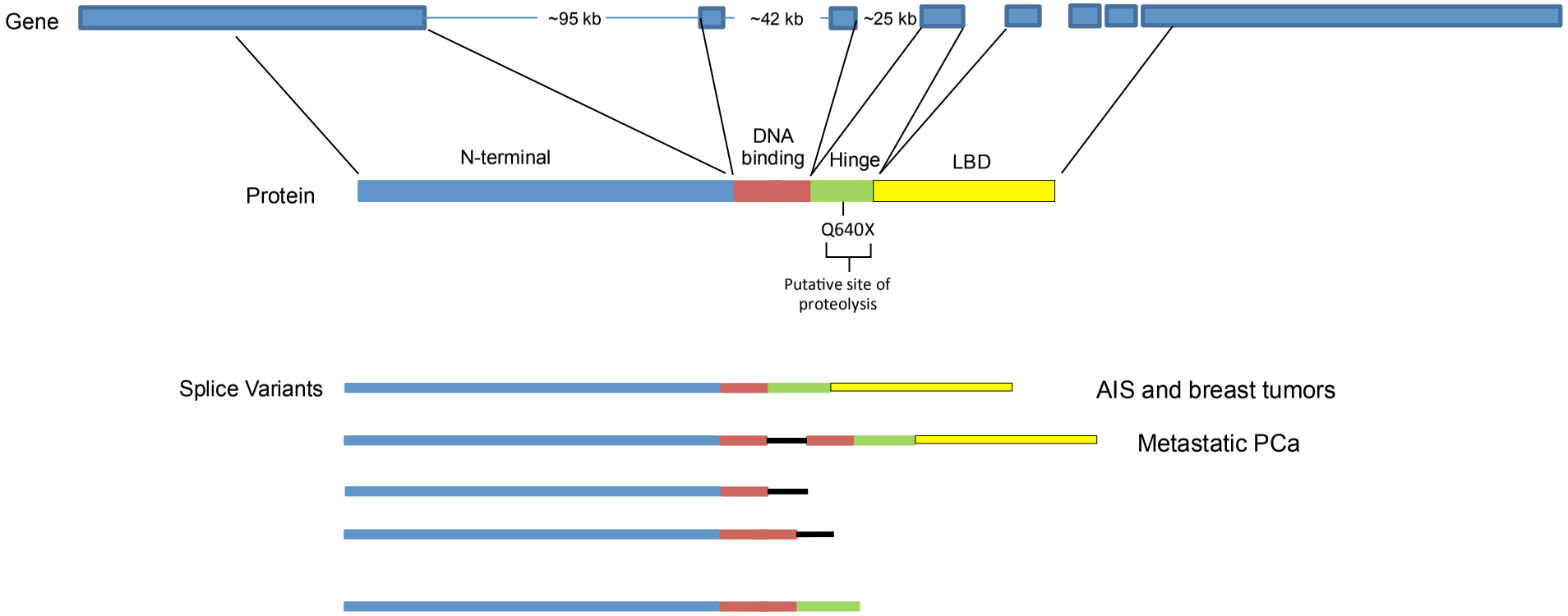
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Protein
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Putative site of proteolysis
Splice Variants
AIS and breast tumors
Metastatic PCa

Potential role of *CHES1*/*FOXN3* as an anti-apoptotic regulator of prostate cancer response to androgen ablative and genotoxic therapies.

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Castration-resistant prostate cancer (CRPC) is highly aggressive and the primary cause of mortality due to prostate cancer (CaP). As a result, the mechanisms underlying progression after androgen ablation are intensely studied. Using LNCaP cells subjected to androgen withdrawal (AW), our initial expression profiling studies identified *CHES1/FOXN3* as a potential molecular mediator of progression by virtue of its *1)* AW-induced pattern of expression and *2)* the ability of RNA interference-mediated *CHES1* silencing to selectively induce apoptosis in the absence of androgen. *CHES1* is a forkhead family member originally identified as a potential DNA damage checkpoint gene. The goal of this work was to better define the role of CHES1 in modulating cell survival during androgen ablation. *In vitro*, *CHES1* message and protein levels were increased significantly in androgen-dependent LNCaP and CWR22Pc cell lines, and this was conserved *in vivo*, as *CHES1* expression was elevated in CWR22 xenografts 6.2-fold at 14 days post-castration. Combined androgen blockade (AW+bicalutamide/Casodex) induced an even higher and dose-dependent level of expression. At the same time, Akt hyperactivation and down-regulation of *BAK1* and *BNIP3* levels were observed, suggesting that AW-induced *CHES1*/*FOXN3* expression might function in maintaining CaP survival via coordinately regulating anti-and pro-apoptotic pathways. We next explored the involvement of CHES1 in p53-mediated apoptosis triggered by mitomycin C (MMC) and adriamycin (Adr). Western blot analysis showed that while levels of Ser15-phosphorylated p53, total p53, and cleaved PARP increased in MMC- or Adr-treated cells, CHES1 expression was suppressed, suggesting that *CHES1* may be a p53-target gene whose repression is necessary for apoptosis mediated by genotoxic stress. In conclusion, our data indicate that CHES1 is an AW-induced gene that maintains CaP cell survival in the absence of androgen. In summary, *CHES1* may be an attractive therapeutic target for augmenting the efficacy of androgen ablation and chemotherapy.

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MOLECULAR TARGETING OF PROSTATE CANCER DURING ANDROGEN ABLATION: INHIBITION OF CHES1

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Background and Objectives: Defining the mechanisms underlying prostate cancer (CaP) survival during androgen withdrawal (AW) and the establishment of castration resistance are critical to the development of more efficacious therapies. We identified FOXN3/CHES1 (Checkpoint suppressor 1) as a molecular mediator of CaP survival during androgen ablation, since antagonism of its function by RNA interference resulted in apoptotic cell death of LNCaP cells selectively in the absence of androgen. The objectives of this project are to 1) better understand the mechanisms of CHES1 gene expression regulation and function, particularly regarding its role in mediating apoptosis resistance during androgen ablation and 2) test the efficacy of CHES1-silencing therapy in preventing the emergence of castration resistance and develop a mechanism-based, non-invasive imaging strategy for monitoring success of the therapy.Brief Description of Methodologies: LNCaP and CWR22Pc cell lines and CWR22 xenografts were utilized as models. AW was simulated by culturing cells in medium containing charcoal-treated FBS or by castration of mice. At the appropriate times after AW or drug treatments, total RNA was isolated and cell lystates were prepared. Transcript levels were measured using real-time quantitative PCR and immunoblot analysis utilized to evaluate the expression of CHES1, BNIP3, and markers indicative of p53 activation and apoptosis. CHES1 promoter activity was monitored with dual-luciferase reporter assays.Results to Date: Our findings demonstrate that CHES1 exhibits an AW-induced expression pattern and is an anti-apoptotic molecule that potentially acts via induction of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and/or down-regulation of the pro-apoptotic Bcl-2 family members BNIP3 and BAK1. In LNCaP cells subjected to androgen deprivation, CHES1 message and protein levels were markedly increased and this was conserved in vivo, as CHES1 expression was elevated in CWR22 xenografts 6.2-fold at 14 days post-castration. Combined androgen blockade (AW+bicalutamide) induced an even higher level of CHES1 expression. Accordingly, CHES1 promoter activity was markedly enhanced by androgen deprivation. CHES1 was originally implicated as a mediator of the DNA damage checkpoint response and our results demonstrate that CHES1 is down-regulated in response to apoptosis-inducing doses of ionizing radiation, adriamycin, and mitomycin C. Interestingly, enforced expression of CHES1 in LNCaP cells conferred an AW phenotype characterized by diminished androgen receptor expression, Akt hyperactivation, and BNIP3 down-regulation. Conclusions: The results demonstrate that CHES1 is an AW-induced gene that

potentially mediates the establishment of an anti-apoptotic context via coordinating PI3K/Akt pathway activation and suppression of BNIP3 expression. In addition, CHES1 down-regulation accompanies, and might be a necessary event, for genotoxic stress to trigger apoptosis. Impact Statement: A critical, clinically-relevant finding is that CHES1 is up-regulated substantially during combined androgen blockade, thereby implicating it as a significant mechanism responsible for resistance to this therapy. Since CHES1 inhibition can convert androgen ablation to a more cytotoxic therapy, these results support the rationale that CHES1 can be exploited as a therapeutic target.

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Molecular targeting of prostate cancer during androgen ablation: inhibition of CHES1/FOXN3

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Abstract

Background and Objectives: Defining the mechanisms underlying prostate cancer (CaP) survival during androgen withdrawal (AW) and the establishment of castration resistance are critical to the development of more efficacious therapies. We identified *FOXN3*/*CHES1* (*Checkpoint suppressor 1*) as a molecular mediator of CaP survival during androgen ablation, since antagonism of its function by RNA interference resulted in apoptotic cell death of LNCaP cells selectively in the absence of androgen. The objectives of this project are to 1) better understand the mechanisms of CHES1 gene expression regulation and function, particularly regarding its role in mediating apoptosis resistance during androgen ablation and 2) test the efficacy of CHES1-silencing therapy in preventing the emergence of castration resistance and develop a mechanism-based, non-invasive imaging strategy for monitoring success of the therapy.

Methodologies: LNCaP and CWR22Pc cell lines and CWR22 xenografts were utilized as models. AW was simulated by culturing cells in medium containing charcoal-treated FBS or by castration of mice. At the appropriate times after AW or drug treatments, total RNA was isolated and cell lysates were prepared. Transcript levels were measured using real-time quantitative PCR and immunoblot analysis utilized to evaluate the expression of CHES1, BNIP3, and markers indicative of p53 activation and apoptosis. CHES1 promoter activity was monitored with dual-luciferase reporter assays.

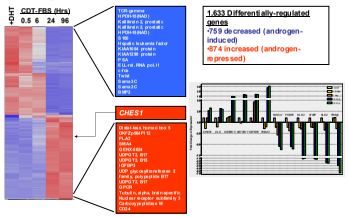
Results to Date: Our findings demonstrate that CHES1 exhibits an AW-induced expression pattern and is an anti-apoptotic molecule that potentially acts via induction of the PI3K/Akt pathway and/or down-regulation of the pro-apoptotic Bcl-2 family members BNIP3 and BAK1. In LNCaP cells subjected to androgen deprivation, CHES1 message and protein levels were markedly increased and this was conserved in vivo, as CHES1 expression was elevated in CWR22 xenografts 6.2-fold at 14 days post-castration. Combined androgen blockade (AW +bicalutamide) induced an even higher level of CHES1 expression. Accordingly, CHES1 promoter activity was markedly enhanced by androgen deprivation. CHES1 was originally implicated as a mediator of the DNA damage checkpoint response and our results demonstrate that CHES1 is down-regulated in response to apoptosis-inducing doses of ionizing radiation, adriamycin, and mitomycin C. Interestingly, enforced expression of CHES1 in LNCaP cells conferred an AW phenotype characterized by diminished androgen receptor expression, Akt hyperactivation, and BNIP3 down-regulation.

Conclusions: The results demonstrate that CHES1 is an AW-induced gene that potentially mediates the establishment of an anti-apoptotic context via coordinating PI3K/Akt pathway activation and suppression of BNIP3 expression. In addition, CHES1 down-regulation accompanies, and might be a necessary event, for genotoxic stress to trigger apoptosis.

Introduction

- Castration-resistant prostate cancer (CaP) is the principal cause of mortality due to this neoplasm.
- To better understand CaP tumor progression, we utilized the LNCaP human androgen-dependent prostate cancer cell line to investigate the survival mechanisms that are engaged during androgen ablation.
- Classic features of androgen ablation are recapitulated in the LNCaP model including: decreased AR protein stability and expression, decreased androgen-responsive gene expression, growth arrest, and neuroendocrine differentiation.
- Similarly to the clinical scenario, the outgrowth of androgen-independent cells is characterized by reinstatement of AR protein expression and signaling.
- Two of the most prominent survival pathways in the LNCaP model are mediated through the AR and PI3K-Akt. In the absence of androgen, the PI3K pathway is hyperactivated and serves as a dominant survival pathway.
- In an effort to define novel molecular mediators of survival during androgen ablation, Affymetrix microarray gene expression profiling was performed on LNCaP cells deprived of androgen for up to 96 hours. We reasoned that genes exhibiting markedly elevated levels of expression might also represent critical survival molecules and novel therapeutic targets.
- *Checkpoint suppressor 1* (*CHES1*)/*FOXN3* was one of the most highly up-regulated genes in response to androgen ablation.
- In this poster, we present the results of our experiments aimed at defining the role of CHES1 in prostate cancer resistance to androgen ablative therapy.

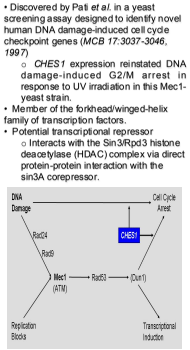
CHES1/*FOXN3* is regulated in an androgen withdrawal-induced, or androgen-repressed, manner.

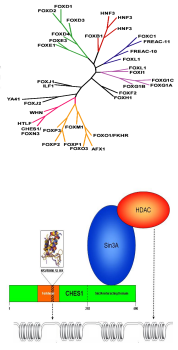


LNCaP cells were cultured in RPMI 1640 medium (w/o phenol red)/10% charcoal/dextran-treated FBS (CDT-FBS) supplemented with DHT (1 nM) or subjected to androgen deprivation for the indicated durations. RNA extracted from each sample was analyzed on Affymetrix HG-U95Av2 GeneChips. Expression data were analyzed by hierarchical clustering (dChip) to identify genes differentially expressed as a response to androgen deprivation. Clusters of genes exhibiting diminished (**blue**) or elevated (**red**) expression patterns are depicted. Samples displaying the median expression for a particular gene are indicated in white, while the magnitude of decreased or increased expression is represented by increasingly darker shades of blue or red, respectively.

Checkpoint Suppressor 1 (*CHES1*)/*FOXN3* background

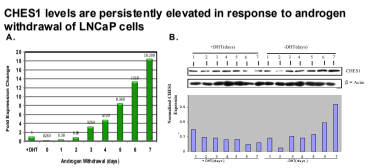
- Discovered by Pati *et al.* in a yeast screening assay designed to identify novel human DNA damage-induced cell cycle checkpoint genes (*MCB 17:3037-3046, 1997*)
  - *CHES1* expression reinstated DNA damage-induced G2/M arrest in response to UV irradiation in this Mec1-yeast strain.
- Member of the forkhead/winged-helix family of transcription factors.
- Potential transcriptional repressor
  - Interacts with the Sin3/Rpd3 histone deacetylase (HDAC) complex via direct protein-protein interaction with the sin3A corepressor.





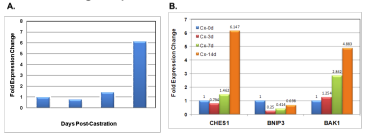
Results

CHES1 levels are persistently elevated in response to androgen withdrawal of LNCaP cells



A) LNCaP cells were cultured in the presence of DHT (1nM, 7 days) or subjected to androgen deprivation for 0-7 days by shifting the cultures to medium supplemented with charcoal/dextran-treated FBS (CDT-FBS). At each time point, the cells were harvested for isolation of total RNA using the RNeasy kit (Qiagen). Subsequently, *CHES1* transcript levels were determined using quantitative RT-PCR (qRT-PCR) with specific TaqMan gene expression assays (Applied Biosystems). All results were normalized to the expression of *GAPDH* in each sample. Fold changes in *CHES1* expression at each time point were calculated relative to that of the DHT-treated sample. ***B)*** LNCaP cells were cultured as the same conditions as above and analyzed by immunoblot analysis for the expression of CHES1 protein (*top panel*). The same blots were also probed for the expression of β-actin as a normalization control. Normalized CHES1 expression levels (*i.e.*, using β-actin) in each sample are depicted by the graph in the bottom panel.

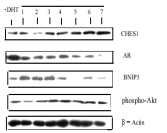
CHES1 gene expression is induced *in vivo* in an experimental model of androgen deprivation.



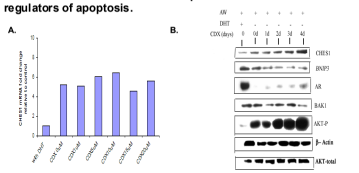
CWR22 xenografts were established in male, nude athymic mice by subcutaneous injection of a suspension of 2.5 x 10^6 cells in Matrigel basement mebrane matrix (BD Pharmingen). Three days prior to injection, each mouse was implanted with a 90-day sustained-release testosterone pellet (12.5 mg). When the tumors reached a size of 0.5-cm^3, androgen ablation was initiated by castration (Cx) of the mice (*i.e.*, bilateral orchiectomy). At 0, 3, 7, and 14 days post-castration, mice were sacrificed and the tumors harvested. Total RNA was prepared and qRT-PCR performed as described in the legend to Figure 2. ***A)*** LNCap cells were stayed in AW medium for 2 days, and then were cultured in the presence of DHT or treated with 0 to 20 μM Casodex for 48 hours.Total RNA was prepared and qRT-PCR performed as described in the legend to Figure 2.. ***B)*** In the same study, qRT-PCR analyses were also performed to examine the expression of the pro-apoptotic Bcl-2 family members *BNIP3* and *BAK1*.

Androgen withdrawal mediates coordinated regulation of CHES1 and apoptosis-regulatory proteins.

LNCaP cells were treated with or DHT (1 nM) or subjected to androgen deprivation (*-DHT*) for 1-7 days followed by preparation of detergent cell lysates. While AW induced a elevated CHES1 and phospho-Akt(S473) levels, it also mediates repression of the pro-apoptotic protein BNIP3.

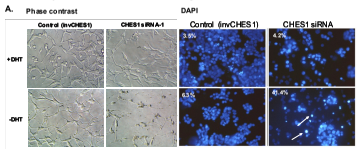


Effect of Casodex treatment on expression of CHES1 and regulators of apoptosis.

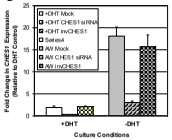


A) LNCaP cells were maintained in AW medium for 2 days, and then cultured in the presence of DHT or treated with 0-20 μM Casodex for 48 hours. Total RNA was prepared and qRT-PCR performed as described in the legend to Figure 2. ***B)*** LNCaP cells were cultured in AW medium for 3 days and then treated with 10 uM Casodex for the indicated times. Immunoblot analyses demonstrated that of AR, BAK1 and BNIP3 expression were down-regulated while CHES1 and phospho (S473)-Akt levels were elevated after Casodex treatment.

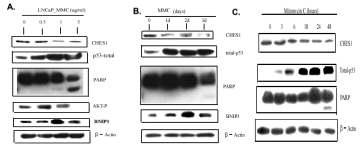
RNAi-mediated silencing of *CHES1* expression induces apoptosis selectively in the absence of androgen.



LNCaP cells were treated with 100 nM CHES1-targeted siRNA (CHES1-Ri-1) in the presence and absence of DHT for 3 days. Oligofectamine-mediated transfection was used for delivery. ***A)*** Morphological changes indicative of apoptosis were evident using standard phase contrast microscopy (*left panels*). Percentage apoptosis was quantitated by enumerating the number of DAPI-stained nuclei exhibiting chromatin condensation and/or fragmentation (*arrows*) using fluorescence microscopy (*right panels*). Percentage apoptosis is indicated in each panel. ***B)*** Effects upon expression were evaluated by quantitative qRT-PCR.

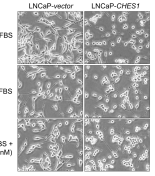


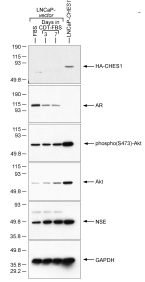
Repression of CHES1 expression is associated with p53-mediated apoptosis in androgen-dependent CaP cells.



The potential involvement of CHES1 in p53-mediated apoptosis triggered by mitomycin C (MMC) was explored. Cells were treated with MMC followed by immunoblot analysis for *1)* the expression of CHES1, p53, BNIP3, and β-actin, *2)* Akt activation, and *3)* PARP cleavage. ***A)*** Dose-response (0-5 μg/ml) and ***B)*** time-course experiments were performed in LNCaP cells followed by immunoblot analysis for the parameters above. ***C)*** A similar time-course experiment was performed with CWR22Pc androgen-dependent CaP cells by treatment with MMC at 1 μg/ml.

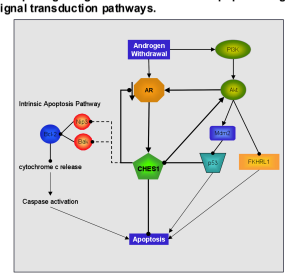
Enforced *CHES1* expression engenders phenotypic and biochemical features characteristic of androgen ablation.





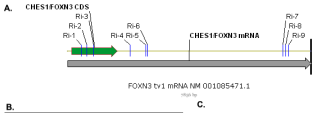
LNCaP-*CHES1* cells were biochemically characterized to evaluate the levels of standard markers, or features, of androgen deprivation. Immunoblot analysis was performed on NP-40 cell lysates from LNCaP-*CHES1* and parental LNCaP cells cultured in the presence of androgen as well as LNCaP cells subjected to androgen ablation for 3 and 7 days.

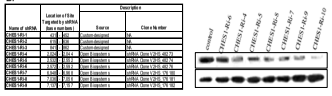
Model depicting integration of CHES1 into apoptosis-regulatory and signal transduction pathways.



Based upon our findings, we have proposed the above model for the mechanism(s) through which CHES1 promotes apoptosis resistance. CHES1 expression in LNCaP cells is repressed by the androgen receptor in the presence of androgen. During androgen ablation, AR signaling ceases, thereby relieving the suppression of *CHES1* transcription, and resulting in elevated CHES1 expression. CHES1 can then potentially antagonize apoptosis in one or more ways. First, persistent CHES1 expression can promote hyperactivation of the PI3K-Akt pathway resulting in sequestration of FKHR-L1 in the cytoplasm, Mdm2-mediated p53 degradation, and up-regulation of Bcl-2. Alternatively, CHES1 might directly repress the expression of genes encoding pro-apoptotic proteins, such as BNIP3 and Bak, manifesting in a an increased threshold to trigger the execution phase (*e.g.*, mitochondrial cytochrome c release, caspase activation) of apoptosis.

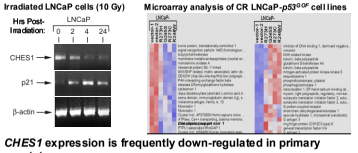
Development of tetracycline-inducible shRNA expression model systems.



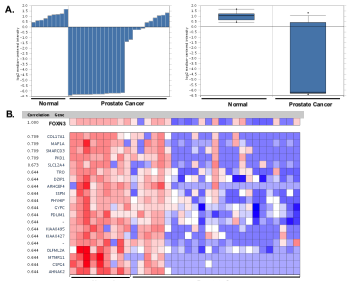


Stable LNCaP *CHES1* knock-down sublines were generated by retroviral expression of *CHES1*-targeting shRNAs. For this, LNCaP cells were infected with pSM2-*CHES1*-Ri amphotropic retroviruses produced in Lin-X-A packaging cells and subsequently selected with puromycin. ***A)*** The *CHES1* mRNA (7,856 bases) is schematically represented. The CDS (green) is indicated as well as the locations of the sites targeted by shRNAs (Ri-1, -2. etc.) used in our experiments. ***B)*** The shRNAs used in this study were either custom-designed to target the CHES1 CDS or were purchased from Open Biosystems. The information for each shRNA is presented in this table, including name, specific location, source, and clone number (if applicable). ***C)*** Western blot analysis illustrating efficiency of RNAi-mediated silencing of CHES1 expression In each subline.

Expression of *CHES1* is down-regulated by p53 activation and following the transition to castration-resistance.



CHES1 expression is frequently down-regulated in primary prostate cancers.



The expression of *CHES1* in clinical prostate cancer samples was surveyed in a microarray dataset (Welsh, JB *et al.* Cancer Res 61:5974-5978, 2001) using the Oncomine web application (https://www.oncomine.org). *CHES1* was down-regulated in 64% (16/25) of the samples relative to its expression in non-malignant prostatic epithelium. ***A)*** *FOXN3*/*CHES1* expression level (signal intensity) in individual samples and as averaged expression in each group are depicted in the bar graph and box plot, respectively. ***B)*** The relative expression of *CHES1* and genes in its coexpression cluster is represented in the heat map.

Summary

- *CHES1*/*FOXN3* represents a critical mechanism of prostate cancer survival during androgen ablation and potential mediator of castration resistance.
  - Expression is induced by androgen deprivation and by anti-androgen treatment.
  - *CHES1* silencing induces apoptosis selectively in the absence of androgen.
- CHES1 may play an important role in maintaining cell survival and inhibiting cancer cell apoptosis during androgen ablation by:
  - Activation of PI3K-Akt signaling.
  - Inhibiting the expression of pro-apoptotic Bcl-2 family members BAK1 and BNIP3.
- p53-mediated suppression of *CHES1* expression might be a critical event in successful propagation of chemotherapy-induced apoptotic signals.
- Taken together, the results suggest that CHES1 can be exploited as a therapeutic target for increasing the efficacy of both androgen ablation and chemotherapy.

Impact Statement

A critical, clinically-relevant finding is that CHES1 is up-regulated substantially during combined androgen blockade, thereby implicating it as a significant mechanism responsible for resistance to this therapy. Since CHES1 inhibition can convert androgen ablation to a more cytotoxic therapy, these results support the rationale that CHES1 can be exploited as a therapeutic target.

Future Directions

- Define the exact position and function of CHES1 in the apoptosis-regulatory network by determining the mechanisms through which:
  - it hyperactivates the PI3K-Akt pathway and suppresses *BNIP3* and *BAK1* expression, and
  - the p53 pathway controls it's expression in response to genotoxic stress, including confirmation of *CHES1* as a p53-target gene.
- Perform *proof-of-principle* xenograft studies in order to demonstrate that antagonism of CHES1 function *in vivo* will induce apoptosis of androgen-dependent prostate cancer cells during androgen ablation and/or prevent the emergence of castration-resistant disease.

Acknowledgements

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Amino acid-mediated mTORC1 activation is a central integration point for androgen receptor and survival signaling in prostate cancer.

Nong Xiang, Shawn M. Purnell, Christopher B. Wee, David L. Boucher, Xu-Bao Shi, Ralph W. deVere White, Jeffrey P. Gregg, Hsing-Jien Kung, and Clifford G. Tepper. UC Davis Cancer Center and Departments of Biochemistry and Molecular Medicine, Urology, Pathology and Laboratory Medicine, UC Davis School of Medicine, Sacramento, CA 95817

The molecular mechanisms underlying prostate cancer resistance to androgen ablation are intensely studied. In the androgen-sensitive LNCaP model, diminished androgen receptor (AR) signaling represents the pivotal response to androgen withdrawal (AW) and mediates survival via hyperactivation of the PI3K-Akt pathway and elevation of Bcl-2 expression. Our results demonstrate that mTOR complex 1 (mTORC1) functions as an integrator of this response in that androgen is required to attain maximal mTORC1 activity and translation initiation complex assembly while AW markedly diminished mTORC1 activity, which consequently led to de-repression of PI3K-Akt signaling and autophagy. Concurrently, AR levels declined due to continued suppression of its translation by residual, androgen-independent, mTORC1 activity; inhibition of this remaining signal with rapamycin completely restored AR expression to levels equivalent to or exceeding that observed in the presence of androgen and recapitulated the biochemical signature of castration-resistant LNCaP-cds sublines, which are defined by elevated AR, phospho-Akt(S473), and Bcl-2 levels. In order to investigate the possibility that androgen might regulate amino acid signaling to mTORC1, we examined the interaction of mTORC1 with Rag GTPases. Notably, androgen markedly enhanced immunocomplex formation of mTOR-raptor with RagB-D heterodimers. Consistent with this finding, the androgen-dependency of this association was bypassed or attenuated by expression of RagB mutants constitutively bound to either GTP or GDP, respectively. In summary, our data demonstrate that *1)* amino acid-stimulated mTOR activity represents a vital regulatory locus of androgen-mediated biology and *2)* aberrant regulation of the AR-mTOR-Akt axis might represent a critical mechanism underlying the transition to castration-resistant disease.
Supported by Department of Defense PCRP Idea Award PC081032 (C.G.T.) and seed funding from the UC Davis Cancer Center.

Amino acid-mediated mTORC1 Activation Is a Central Integration Point for Androgen Receptor and Survival Signaling in Prostate Cancer

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Abstract

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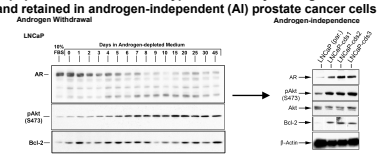
Introduction

- Prostate carcinoma (CaP) is the second most common malignancy occurring in men. In 2009, approximately 235,000 new cases of CaP were diagnosed and nearly 27,000 men died from the disease.
- Androgen is a dominant mediator of CaP biology.
  - It drives growth, survival, and epithelial differentiation of both normal and malignant prostatic epithelial cells.
- Androgen ablative therapy exploits this feature to suppress androgen-AR signaling and is very effective in mediating regression.
  - However, this is only palliative and the disease typically recurs as castration-recurrent/ resistant prostate cancer (CRPC), or androgen-independent (AI) CaP.
- A prominent mechanism underlying CRPC is the reinstatement of AR signaling.
  - Signified clinically by elevated serum PSA (biochemical failure).
  - AR transcriptional program is restored, albeit incompletely.
- A major reason for the failure of this therapy to cure CaP is that only a sub-population of CaP cells are killed by androgen ablation.
  - This is in marked contrast to normal prostate in which castration induces massive apoptosis of terminally-differentiated luminal cells.
- Taken together, the survival of cancerous prostate cells is supported by both androgen-dependent and androgen-independent mechanisms.

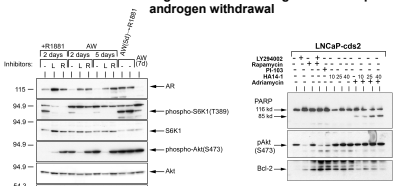
Results

Anti-apoptotic mechanisms are hyperactivated by androgen withdrawal and retained in androgen-independent (AI) prostate cancer cells



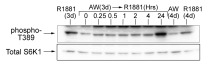
❖Androgen withdrawal of androgen-dependent/sensitive LNCaP cells: Diminished AR signaling hyperactivates PI3K-Akt signaling and enhances Bcl-2 expression.
❖Androgen independent LNCaP-cds cell lines: Despite reinstatement of AR expression and signaling, both survival mechanisms persist and are further de-regulated.
❖PI3K-Akt and Bcl-2 not only represent androgen-independent survival mechanisms, but anti-apoptotic mediators negatively regulated by AR signaling.

mTORC1 acts as an androgen sensor and integrates the response to androgen withdrawal



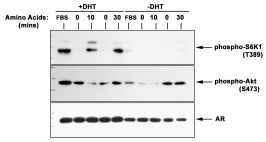
❖The goal of this experiment was to investigate the regulation of mTOR by androgen signaling and its potential influence upon AR and PI3K-Akt signaling.
❖Androgen was required to maintain complete activation of mTOR.
❖Conversely, AW mediated a marked diminution in phospho-S6K1(T389) levels.
❖However, low level, residual mTOR activity remained in the absence of androgen.
❖The importance of androgen-independent (AI) mTOR activity to mediating the effects of AW is underscored by the finding that AR expression was completely restored by mTOR inhibition with LY294002 (*L*) or rapamycin (*R*).
❖PI3K/Akt hyperactivation is a result of AW- or rapamycin-mediated mTORC1 inactivation.
❖Although mTOR-S6K1 exhibited reversible activation by androgen, AW-induced PI3K-Akt activation was refractory to repression by subsequent androgen treatment.
❖Bcl-2 expression is elevated in response to mTORC1 inhibition (*right panel*)

Androgen-mediated mTOR activation proceeds with slow kinetics.



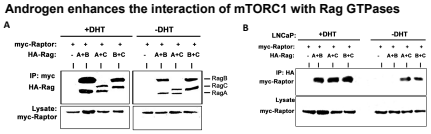
❖Time-course experiments further demonstrated that while R1881 induced partial mTOR reactivation within 15 minutes, full reinstatement of phospho-S6K1(T389) levels proceeded with slow kinetics and required 24 hours.

Androgen-AR signaling is required for amino acid-mediated mTORC1 activation.



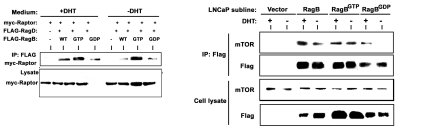
❖LNCaP cells were cultured in the presence or absence of DHT for 72 hours prior to amino acid starvation 50 minutes and subsequent re-addition of amino acids for 10 or 30 minutes.
❖Amino acid starvation resulted in a complete loss of mTORC1 activity, which was restored by amino acid stimulation in the presence, but not absence of androgen.

Androgen enhances the interaction of mTORC1 with Rag GTPases



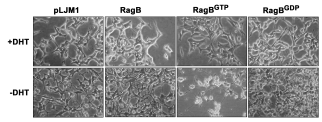
❖Co-transfection and co-IP experiments demonstrated that Rag heterodimers interact with mTORC1 via raptor in an androgen-dependent manner.
A) LNCaP cells were co-transfected as described above and cultured for 72 hours in the presence (*+DHT*) or absence (*-DHT*) of DHT. Co-IP analysis was performed by precipitation with anti-myc followed by immunoblotting for co-precipitated Rag complex components with anti-HA (*upper panel*) and control for raptor expression with anti-myc (*lower panel*).
B) The same experiment as described in ***A*** was conducted, except co-IP analysis was performed by IP with anti-HA and subsequent analysis for Raptor (anti-myc) in the immune complex (*upper panel*).

Enforced expression of a RagB^{GTP} mutant bypasses the requirement of androgen for the interaction of mTOR with RagB/D heterodimers.

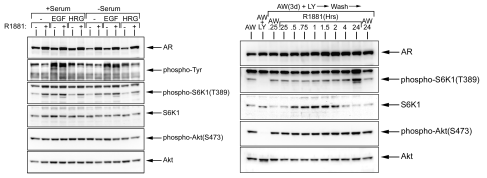


❖Left panel: LNCaP cells were co-transfected with myc-raptor, RagD-WT, and different forms of RagB (WT, 54L/GTP, 99L/GDP). Cells were then cultured in the presence or absence of DHT followed by IP with anti-FLAG and immunoblot analysis for myc-raptor.
❖Right panel: A similar experiment to that described above was performed using LNCaP cells stably expressing RagB, RagB^{GTP}, or RagB^{GDP}.

Stable expression of RagB^{GTP} compromises the survival of LNCaP cells in the absence of androgen.

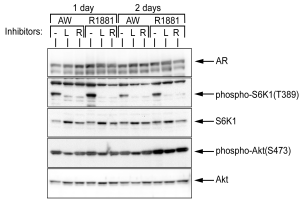


Polypeptide growth factors activate mTORC1 independently of androgen.



❖We hypothesized that residual, AI mTOR activity was driven by serum-derived growth factors. To address this, we completely inhibited mTOR and then determined the capacity of serum and androgen to reinstate its activity.
❖Basal, AI S6K1 phosphorylation was completely suppressed by PI3-K inhibition (*AW+LY*), but was fully restored within 15 minutes of removal of the inhibitor (*AW 0.25*). Androgen-stimulated mTOR activity was not apparent until 4 hours after the addition of R1881 and peaked at 24 hours (*AW+LY*). In contrast, phospho-S6K1 levels were unchanged after the same duration in the absence of androgen (*AW 24*).
❖These results demonstrate that serum-derived growth factors can rapidly activate mTOR independently of androgen signaling and that androgen enhances this signal, but requires longer term signaling.

Disruption of the AR-mTORC1-Akt signaling axis represents a critical molecular event in the transition to CRPC.



❖LNCaP-cds cell lines are characterized by having greatly elevated levels of AR expression and phospho-Akt(S473) compared to the parental LNCaP.
❖Like LNCaP, R1881 treatment increased T389 phosphorylation of S6K1.
❖In marked contrast to LNCaP, Akt was not negatively-regulated by synthetic-androgen-induced mTOR activation:
➢ Inhibition of mTORC1 with rapamycin did not induce higher levels of Akt activation.
➢Androgen-induced mTOR activation did not repress Akt, but was associated with increased Akt(S473) phosphorylation.

Summary of Key Findings

- **mTORC1 functions as a a critical sensor of androgen signaling and integrates AW-induced PI3K-Akt hyperactivation, Bcl-2 up-regulation, and AR down-regulation.**
  - Androgen and growth factors independently activate mTOR via AR and PI3K, respectively.
  - The results suggest that there is an AR-mTORC1-PI3K/Akt signaling axis. In each case, feedback-inhibitory loops are established by mTOR-mediated suppression of AR expression and PI3K activation.
- **Androgen-AR signaling is required to maintain full mTORC1 activity in androgen-sensitive LNCaP cells.**
- **Amino acid signaling to mTORC1 is the prominent pathway influenced by androgen.**
  - The ability of amino acids to activate mTORC1 is markedly diminished during androgen deprivation.
  - In contrast, serum and polypeptide growth factors can stimulate mTORC1 in both the presence and absence of androgen.
- **Androgen regulates the interaction of mTORC1 with Rag heterodimers.**
- **The data suggest that androgen regulates the recruitment of mTORC1 to the surface of the lysosome, thereby connecting androgen deprivation with the induction of autophagy.**
  - Taken together with the above findings, this is consistent with the model described for amino acid-mediated activation of mTORC1 via interaction with the Rag GTPases (Sancak, Y. *et al.* Science, 320:1496, 2008; Sancak, Y. *et al.* Cell, 141:290, 2010).
- **Although enforced expression of Rag^{GTP} can bypass the requirement for androgen to mediate mTORC1-RagB/D interactions, it does not support androgen-independent growth.**
- **Disruption of the AR-mTORC1-PI3K/Akt axis contributes to AW-induced AR down-regulation, Akt hyperactivation, and Bcl-2 up-regulation:**
  - Growth factor driven activation of the androgen-independent mTOR component is responsible for driving down AR expression in the absence of androgen.
  - Loss of the androgen-sensitive amino acid-mTOR component results in de-repression of PI3K-Akt signaling.

Acknowledgements

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Identification of *checkpoint suppressor* 1 (*CHES1*)/FOXN3 as an androgen withdrawal-induced gene mediating neuroendocrine differentiation and survival of prostate cancer cells.

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Running Title: *CHES1* promotes NED and survival of prostate cancer

Key words: microarray, androgen independence, *CHES1*, neuroendocrine differentiation, apoptosis, RNA interference

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ABSTRACT

The molecular mechanisms underlying the development of prostate cancer (CaP) resistance to androgen-ablative therapy are intensely studied. We performed expression profiling of androgen-deprived LNCaP cells in order to identify genes vital to the survival and eventual outgrowth of cells challenged by androgen ablation. Hierarchical clustering of the microarray data organized 159 differentially expressed genes into two clusters of androgen withdrawal (AW)-repressed and AW-induced genes. *Checkpoint suppressor 1* (*CHES1*) was identified in the latter cluster as having low expression in the presence of androgen, but marked induction (3.25-fold) following 96 hours of androgen withdrawal. *CHES1* is a forkhead transcription factor discovered in a yeast screen for potential human DNA damage checkpoint genes. However, its expression was strongly repressed by ionizing radiation-induced p53 activation. RNA interference-mediated *CHES1* silencing inhibited the basal growth of LNCaP cells and induced apoptosis in the absence of androgen. LNCaP sublines stably expressing physiologically relevant levels of the HA-tagged CHES1 protein (LNCaP-*CHES1*) were also generated. These possessed a distinctive neuroendocrine (NE) phenotype, evidenced by rounded cell bodies and extension of neuritic processes, and proliferated at an attenuated rate compared to LNCaP-*vector* cells. Biochemical characterization of LNCaP-*CHES1* cells demonstrated nuclear-localized expression of CHES1, diminished androgen receptor (AR) expression, elevated phosphorylated Akt levels, and increased neuron-specific enolase levels. In summary, our results demonstrate that *CHES1* is an AW-induced gene that promotes CaP survival in the absence of androgen and is a potential, dominant mediator of the androgen-ablated phenotype by suppressing AR signaling and promoting NE differentiation.

INTRODUCTION

Androgen ablation is the first-line therapy for metastatic, hormone-dependent prostate cancer (CaP) and exploits the obligatory role of testicular androgens for the proliferation, differentiation, and survival of nonmalignant prostatic exocrine epithelial cells as well as cancers. This is only palliative, as hormone-refractory, or androgen-independent (AI), disease typically recurs after eighteen to twenty-four months and accounts for the 20% mortality rate due to this neoplasm (1). As a result, the mechanisms underlying the transition from androgen dependence to androgen independence are the subject of intense investigations.

The LNCaP androgen-dependent prostate carcinoma cell line has been valuable for studying the action of androgen and modeling human CaP *in vitro* and *in vivo*. Key consequences of androgen ablation in this model are AR pathway inactivation, growth arrest, neuroendocrine differentiation (NED) (2), and the engagement of mechanisms antagonizing apoptosis (3). The androgen receptor (AR) acts as the pivotal effector of androgen deprivation. Removal of its ligand simultaneously decreases AR-mediated transcriptional regulation and effectively eliminates the AR pathway by triggering a marked reduction in AR protein levels (4) via destabilization (5) and proteasome-mediated degradation (6). As a result, growth arrest rapidly ensues and is highlighted by Rb-mediated accumulation of cells in G1 and a corresponding, dramatic reduction in S-phase cells undergoing DNA replication (7).

Neuroendocrine transdifferentiation occurs simultaneously with growth arrest and is an obvious feature of androgen-deprived LNCaP cultures by virtue of striking morphological changes (2, 3). The role of androgen signaling in actively repressing NED has been supported by the ability of RNA interference (RNAi)-mediated AR gene silencing to induce phenotypic and biochemical markers of NED (8). These cells possess numerous secretory granules containing a variety of neurotrophic factors including chromogranin A, neuron-specific enolase (NSE), neurotensin, serotonin, somatostatin, and gastrin-releasing peptide/bombesin (9). While nonmalignant NE cells are normally present in the epithelium of the prostate gland, many studies have underscored the critical role of transdifferentiated, malignant NE cells and their products in mediating tumor progression via the ability to promote survival, androgen-independent proliferation, AR activation, and metastasis (9, 10).

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a dominant survival factor for LNCaP cells during androgen deprivation. PI3K signaling is deregulated in this model due to mutational inactivation of the PTEN lipid phosphatase and a consequential accumulation of 3-phosphorylated phosphatidyl inositides. Androgen withdrawal hyperactivates the pathway and its inhibition by pharmacological inhibitors (*e.g.*, LY294002, wortmannin) or ectopic PTEN expression induced apoptosis specifically under these conditions (3, 11). Downstream anti-apoptotic mechanisms of PI3K/Akt signaling include Akt-mediated inhibition of FKHR/FKHRL1 transcription of pro-apoptotic genes and activation of mammalian target of rapamycin (mTOR) and hypoxia-inducible factor-1α (HIF-1α) target genes (12). Additionally, transcriptional activation of Bcl-2 by NF-κB and cAMP-response element-binding protein (CREB) has been linked to the PI3K pathway (13, 14). Notably, AI LNCaP sublines selected by outgrowth in hormone-free medium are characterized by elevated Bcl-2 expression (15, 16).

Genome-wide expression profiling (*e.g.*, SAGE, microarray) has contributed greatly to our understanding of the mechanisms underlying the development of hormone-refractory prostate cancer. This approach has clearly demonstrated the reinstatement of the AR transcriptional program as a consistent feature of AI tumors (17, 18) and the significance of AR overexpression in resistance to hormone therapy (19). Elevated expression of genes encoding PI3K/Akt/mTOR pathway components has also been implicated in androgen independence (18). Androgen-repressed genes significantly influence the transition to androgen independence as they contribute to the androgen withdrawal phenotype and to survival in the absence of androgen.

De-repression of the UDP-glucuronosyltransferase (UGT2B7/15/17) genes further suppresses AR signaling by catalyzing the inactivation and elimination of remaining 5α-dihydrotestosterone (5α-DHT) via glucuronidation (20). At the same time, resistance to androgen ablation is mediated by up-regulation of the anti-apoptotic gene clusterin (testosterone-repressed prostate message 2) (21); conversely, clusterin antisense oligodeoxynucleotides induced apoptosis of Shionogi tumors cells and delayed recurrence (21). Accordingly, the identification and targeting of anti-apoptotic genes up-regulated during androgen ablation is of substantial interest for the treatment of prostate cancer (21).

In this manuscript, we describe the identification of *CHES1* by microarray as a gene induced by androgen withdrawal in LNCaP prostate cancer cells. *CHES1* was discovered by Pati and co-workers as a forkhead/winged-helix transcription factor that complemented defective DNA damage checkpoint pathways in yeast (22). We have extended their findings to characterize the role of *CHES1* in a mammalian system. RNAi experiments demonstrated that CHES1 contributed to the basal proliferation of LNCaP and was critical to survival in the absence of androgen. In contrast, enforced expression of *CHES1* inhibited growth of LNCaP and induced the acquisition of a NE phenotype. Biochemical characterization of the LNCaP-*CHES1* subline demonstrated constitutive, nuclear localization of CHES1 and features of androgen withdrawal, such as significantly reduced AR expression and elevated serine 473-phosphorylated Akt and NSE levels.

MATERIALS and METHODS

Cell Lines and Culture.

LNCaP prostate adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 100 U/ml penicillin-100 μg/ml streptomycin at 37°C in a humidified environment of 5% CO_2 in air. LNCaP-*CHES1* sublines were maintained in the same medium under antibiotic selection by supplementation with GENETICIN (G418 sulfate, 400 μg/ml; Invitrogen Life Technologies, Carlsbad, CA). To simulate conditions of androgen ablation, cultures were shifted into RPMI 1640 medium (without phenol red) supplemented with 10% charcoal/dextran-treated fetal bovine serum (CDT-FBS; HyClone). Androgen-independent LNCaP-cds cell lines have been described (16) and were maintained under conditions of chronic androgen deprivation in the medium described above .

Reagents.

5α-DHT was purchased from Sigma Chemical Company (St. Louis, MO). Wortmannin was purchased from Calbiochem (San Diego, CA). Mouse monoclonal antibodies against the androgen receptor (clone AR 441, Ab-1) and NSE (clone E27, IgG1) were purchased from Lab Vision Corporation, (Fremont, CA). Rabbit polyclonal anti-phospho(Ser473)-Akt and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Epitope tag antibodies used in this study included mouse monoclonal anti-HA (HA.11, clone 16B12) purchased from Covance Research Products (Berkeley, CA) and anti-c-myc mouse monoclonal antibody (clone 9E10, IgG1, kappa) from Roche Applied Science (Indianapolis, IN). Rabbit polyclonal anti-phospho-FKHRL1 (Thr 32) and anti-FKHRL1 antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The mouse monoclonal antibody against GAPDH (clone 6C5) was obtained from Chemicon International (Temecula, CA).

Immunoblot Analysis.

NP-40 lystates were prepared from cell cultures and analyzed by immunoblot analysis according to standard protocols as previously described (23).

Oligonucleotide Microarrays.

Human Genome U95A (HG-U95Av2) GeneChip arrays were purchased from Affymetrix (Santa Clara, CA) and provide coverage of 12,599 RNA messages. Each transcript is represented by a probe set consisting of 10 different perfect match probes (25-mers) and a corresponding set of mismatch probes. Complete design and sequence information for each probe set are available at the NetAffx website (http://www.affymetrix.com/analysis/index.affx).

Microarray Gene Expression Profiling and Data Analysis.

GeneChip® expression analysis steps including RNA isolation, cDNA synthesis, preparation of biotin-labeled target cRNA, hybridization, washing, staining, and scanning were performed using standard protocols and reagents described by the manufacturer and as previously described (16). Scanned chip images were scaled to an average hybridization intensity of 125 with Affymetrix Microarray Suite (MAS) software algorithms. Feature extraction, data normalization, comparison analysis, and hierarchical clustering were performed using DNA-Chip Analyzer (dChip) (24). Probe set signal intensities were generated using the perfect match/mismatch model and the data was filtered to select for transcripts having a minimum normalized probe intensity value of 80 and a fold change in expression of $\geq$2.5 at the 96-hour

time point compared to that of LNCaP cells cultured in the presence of androgen. The hierarchical clustering algorithm was utilized to organize the expression data into sets of genes displaying similar expression patterns across the time course of androgen withdrawal.

Analysis of the *CHES1* Genomic Locus.

The exon/intron organization of the CHES1 gene was determined by BLAST analysis of the human genome using the 1,473-bp CHES1 cDNA sequence (NM_005197). The corresponding genomic contig (NT_026437.10|Hs14_26604:69517542-69830120) from chromosome 14 was downloaded and mined for androgen response elements (AREs) and regions (ARRs) using a composite of ARE/ARR consensus sequences [(A/G)(G/T)A(A/T)C(A/T/G)nnn(A/T/G)G(T/A/C)(T/A)(C/A)(T/G/C)] with GeneTool version 1.0 software (BioTools Incorporated, Edmonton, Alberta, Canada). Human genome resources were provided by the National Center for Biotechnology Information (Bethesda, MD).

RNA Isolation and RT-PCR Analysis.

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was prepared from 5 μg total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo$(dT)_{12\text{-}18}$ primer as previously described (23). PCR reactions were performed with custom-synthesized primers (Integrated DNA Technologies, Coralville, IA). Sequences of the primers are as follows. CHES1 (CHES1 1002.For 5′-TCACAACTACAGCAGTGCCAAGTC-3′ and CHES1 1367.Rev 5′-GCTTCTTTCATCTCCTCATCATCG-3′), TUBA3 (TUBA3_100.For 5′-ATGCGTGAGTGCATCTCCATCCAC-3′ and TUBA3_624.Rev 5′-GGGCGCCGGGTAAATAGAGA-3′), GAPD (GAPD 212.For 5′-GAAATCCCATCACCATCTTCCAG-3′ and GAPD 524.Rev 5′-ATGAGTCCTTCCACGATACCAAAG-3′), AR (hAR 2756.For 5′-CCCATTGACTATTACTTTCCACCCC-3′ and hAR 3490.Rev 5′-TTGAGAGAGGTGCCTCATTCGGAC-3′), and $p21^{Cip1/Waf1}$ (p21CIP1_139.For 5′-GAGCGATGGAACTTCGACTTTG-3′ and p21CIP1_491.Rev 5'-GGCTTCCTCTTGGAGAAGATCAG-3′). PCR reactions were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) under the following conditions: 94°C, 5 minutes; 30 cycles (94°C, 30s; 56°C[$p21^{Cip1/Waf1}$]/57°C[GAPD]/59°C[TUBA3]/60°C[CHES1, AR], 30s; 72°C, 60s); 1 cycle (72°C, 2 minutes). Amplification products were analyzed on 1.0% or 1.5% TAE-agarose gels and photographed under UV illumination.

RNA Interference.

Small interfering RNA duplexes (siRNAs) targeting *CHES1* were designed using standard parameters (25) and custom-synthesized (Dharmacon Research, Lafayette, CO). The location of the targeted transcript regions (*i.e.*, relative to the translation start site) and siRNA duplex sequences are as follows. The CHES1-Ri-1 siRNA targeted nucleotides 412-432; sense: 5′-GGAUAUCUACAACUGGAUCdTdT-3′, antisense: 5′-GAUCCAGUUGUAGAUAUCCdTdT-3′. CHES1-Ri-2 siRNA targeted nucleotides 421-441; sense: 5′-CAACUGGAUCUUGGAACAUdTdT-3′, antisense: 5′-AUGUUCCAAGAUCCAGUUGdTdT-3′. CHES1-Ri-3 siRNA targeted nucleotides 993-1013; sense: 5′-AGGAGGAUCACAACUACAGdTdT-3′, antisense: 5′-CUGUAGUUGUGAUCCUCCUdTdT-3′. An RNA duplex sequence targeting nucleotides 153-175 of the firefly (*Photinus pyralis*) luciferase gene in the pGL-2 Control reporter vector (accession #: X65324) was used as a control for non-specific siRNA effects (26).

For analysis of RNAi-mediated suppression of gene expression, cells were seeded into 6-well plates (2.5 x 10^5 cells/well) and transfected in serum-containing medium (without antibiotics) with 100 nM small interfering RNA duplexes using Oligofectamine Reagent (Invitrogen, Carlsbad, CA) according to the protocol described by Harborth and co-workers (26). RNA was isolated after 48 hours of treatment for RT-PCR analysis. For cell proliferation assays, cells were seeded into 96-well plates (1 x 10^4 cells/well) in the presence (FBS) or absence (CDT-FBS) of androgen and transfected the following day. After 72 hours, relative cell proliferation was determined using the MTS cell proliferation assay reagent (Promega, Madison, WI).

Construction of CHES1 Mammalian Expression Plasmids.

The full-length CHES1 coding region was generated by reverse transcription and high fidelity PCR amplification (Expand High Fidelity PCR System, Roche Applied Science, Indianapolis, IN) of total RNA prepared from LNCaP cells androgen-deprived for seven days. Gel-purified amplicons were cloned into pCR2.1-TOPO by TOPO TA cloning (Invitrogen, Carlsbad, CA) and sequence integrity confirmed by automated DNA sequencing (Davis Sequencing, Davis, CA). For expression with a twelve-amino acid hemagglutinin (HA) epitope tag (MGYPYDVPDYAS), the insert generated with CHES1_133_Cpo.For (5´-CGGTCCGATGGGTCCAGTCATGCCTCCCAG-3´) and CHES1_1605_ Xho.Rev (5´-CTCGAGTTAATTTTTTGTGGTTTCCTTTTGCTC-3´) primers was subcloned into *Cpo*I + *Xho*I-digested pcDNA3.1-CMV-HA-Neo(+)—modified from pcDNA3.1(+) (Invitrogen, Carlsbad, CA). For expression of CHES1 with a carboxyl-terminal *myc*-His epitope, the insert generated with CHES1_130 _KS.For (5´-GCCACCATGGGTCCAGTCATGCCTCCCAG-3´) and CHES1_1602_Xba.Rev (5´-TCTAGAATTTTTTGTGGTTTCCTTTTGCTC-3´) primers was subcloned into *Not*I + *Xba*I-digested pcDNA3.1(+)/*myc*-His A (Invitrogen, Carlsbad, CA).

Establishment of LNCaP Sublines Stably Expressing CHES1.

LNCaP cells were transfected with pcDNA3.1-CMV-HA-*CHES1* or pcDNA3.1(+)-*CHES1*-*myc*-His expression constructs using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Stably-transfected clones and pools were selected by culture in the presence of GENETICIN antibiotic (400 μg/ml).

RESULTS

LNCaP Cells Subjected to Androgen Withdrawal Exhibit Features Typical of Androgen Ablation.

In order to better understand the mechanisms responsible for the survival of prostate cancer cells during androgen ablation, we simulated this modality *in vitro* using the LNCaP human androgen-dependent prostate cancer cell line subjected to androgen deprivation as a model system. Levels of expression or activation of selected proteins associated with AR signaling were evaluated by immunoblot analysis of NP-40 lysates prepared from cells harvested one to forty-five days after androgen withdrawal. As shown in **Figure 1A**, androgen deprivation triggered a rapid and marked reduction in AR protein levels and a corresponding decrease in the expression and secretion of prostate-specific antigen (PSA) (23). Although this treatment does not induce apoptosis (*data not shown*), it induces growth arrest and transdifferentiation of LNCaP cells into a neuroendocrine (NE) phenotype (2). This was verified by biochemical and morphological criteria, such as increased NSE expression **(Fig. 1A),** rounding of the cell bodies, and extension of neuritic processes **(Fig. 1B)**. A marked and persistent increase in the levels of activated Ser473-phosphorylated Akt/PKB was also observed **(Fig. 1A)**. In summary, androgen deprivation promotes the appearance of classic features of androgen ablation triggered by attenuated AR pathway signaling resulting in NED, growth arrest, and PI3K/Akt pathway hyperactivation supporting survival of this PTEN-deficient model (3).

Identification of *Checkpoint Suppressor 1* (*CHES1*) as an Androgen Withdrawal-induced Gene by Expression Profiling of LNCaP Cells.

Microarray gene expression profiling was utilized to identify androgen-regulated genes that might support survival and mediate the appearance of one or more features accompanying androgen ablation. RNA was isolated from cells either left untreated in the presence of 1 nM DHT or deprived of androgen for 0.5, 6, 24, and 96 hours followed by analysis of approximately 12,599 transcripts using Affymetrix HG-U95Av2 GeneChip oligonucleotide arrays. Differentially expressed genes and groups, or clusters, of genes displaying similar expression patterns were identified by comparison analysis and hierarchical clustering (24). Since we hypothesized that the genes most likely to impact upon cell physiology during chronic androgen deprivation would be those exhibiting the greatest and persistent changes, our analysis was focused to select only those genes that were differentially expressed by at least $\geq$2.5-fold at the 96-hour time point. This yielded a set of 159 genes divided into two major clusters of 78 up-regulated and 81 down-regulated genes exhibiting changes ranging from 2.72- to 134.53-fold and –2.69- to -68.24-fold, respectively **(Supplementary Tables 1 and 2, Fig. 2A)**. The efficacy of androgen deprivation in this experiment was validated by *1)* the attenuation of androgen-induced gene expression, such as PSA, kallikrein 2, NKX3.1, and TCR gamma alternate reading frame protein and by *2)* the induction of typically androgen-repressed genes including α3-tubulin, insulin-like growth factor binding protein 3, UGT2B17 (20), and clusterin (27, 28) **(Fig. 2B)**.

In these experiments, *checkpoint suppressor 1* (*CHES1*; FOXN3) was identified as an androgen withdrawal-induced gene, exhibiting 2.65- and 3.25-fold induction of expression following 24 and 96 hours of androgen deprivation, respectively **(Supplementary Table 1, Fig. 2A)**. Compared to several other androgen-regulated genes, the magnitude and kinetics of expression was most similar to that displayed by clusterin **(Fig. 2B)**. RT-PCR analysis validated these findings **(Fig. 2C)** and also demonstrated that *CHES1* was expressed at very low levels in androgen-independent LNCaP-cds cell lines derived by selection under long-term androgen-deprivation (16). These data demonstrate that *CHES1* expression is up-regulated in androgen-dependent

LNCaP cells during acute androgen withdrawal, but returns to basal levels after reinstatement of the AR pathway in androgen-independent sublines.

We pursued the role of *CHES1* in CaP biology for several reasons. In addition to its prominent expression in response to androgen deprivation, CHES1 might serve a pivotal function in mediating androgen withdrawal-induced growth arrest and survival; It was originally identified by virtue of its ability to reinstate a G2/M DNA damage checkpoint in several mutant yeast strains, thereby suppressing the lethality of those checkpoint defects (22). Additionally, *CHES1* is a member of the forkhead/winged-helix family, suggesting it has the capacity to influence one or more pathways via its transcriptional targets.

CHES1 Expression Is Potentially Suppressed by p53 Activation.

Since *CHES1* complemented multiple DNA damage checkpoint pathway defects in yeast (22), we hypothesized that CHES1 might function as an effector molecule in a pathway unique to mammalian cells, namely the p53 pathway. To investigate this, *CHES1* expression was analyzed in LNCaP cells exposed to ionizing radiation (IR). As shown in **Fig. 2D**, γ-irradiation (10 Gy) was successful in mediating p53 activation, as $p21^{Cip1/Waf1}$ expression was rapidly induced by two hours and peaked at four hours. In contrast, the low, basal expression of CHES1 was further down-regulated. In summary, the data suggest that *CHES1* is a primary or secondary p53 target gene.

Structure of the _CHES1_ Genomic Locus

To gain insight into sequence determinants responsible for the androgen-regulated expression of *CHES1*, we interrogated the genomic locus for the presence of AREs. *CHES1* resides on chromosome 14q24.3-q31 (NCBI, (22)). A genomic BLAST using the 1,473-bp *CHES1* cDNA sequence revealed that it is encoded by six exons spanning approximately 250 kbp **(Fig. 3A)**. During the cloning of the full-length *CHES1* cDNA sequence from LNCaP RNA, we independently identified *CHES1*β, an isoform of *CHES1* that lacks exon 4 and was presumably generated by alternative splicing **(Fig. 3A)**. The mouse ortholog of *CHES1*, *Mus musculus Ches1*, is located on chromosome 12 and encodes a transcript similar to the *CHES1*β isoform. While a full-length transcript similar to *CHES1*α is encoded by *Mm similar to CHES1* on chromosome X, it is now classified as a pseudogene.

The 312-kbp contig containing the *CHES1* gene, including 31.2 kbp of the 5´- and 3´-untranslated regions, was scanned for consensus AR DNA binding sites using a consensus ARE motif derived from a composite of published sequences (29, 30). This revealed thirty-three potential AREs **(Fig. 3B)**. Although the presence of androgen strongly represses *CHES1* expression, we did not identify any sites in the proximal promoter or enhancer regions of the gene. However, intron 3 contained seventeen AREs, most of which (13/17) are concentrated to its distal region. In summary, transcriptional repression of *CHES1* by androgen is potentially mediated by the binding of the AR to sequence motifs in intron 3 rather than to *cis*-regualtory elements in the 5´-untranslated region of the gene.

RNAi-mediated Silencing of _CHES1_ Expression Induces Growth Inhibition and Apoptosis in the Absence of Androgen.

In order to determine the potential contribution of CHES1 to the maintenance of LNCaP cell growth and viability, the effects of CHES1 silencing were evaluated. For this, cells were transfected with siRNA duplexes, cultured in the presence or absence of androgen for three days, and relative proliferation rates were determined by MTS assays. Treatment with each of the CHES1 siRNAs resulted in growth inhibition **(Fig. 4A)**. In the presence of androgen, this ranged from 27-38% when compared to the growth of cultures treated with a control siRNA

targeting luciferase. In the absence of androgen, inhibition reached 45% with the CHES1-Ri-1 siRNA. Light microscopic examination revealed that apoptosis was induced by this duplex specifically in the absence of androgen as evidenced by the appearance of distinctive features, such as cellular fragmentation and condensation of cytoplasmic contents **(Fig. 4B,** *compare CHES1-Ri-1 panels, -DHT and +DHT***)**. The efficacy and specificity of the CHES1-Ri-1 was demonstrated by its ability to reduce *CHES1* expression by approximately 70% and by the absence of effects upon a non-targeted gene (*e.g.*, AR), respectively **(Fig. 4C)**.

CHES1 Is Expressed as a 56-kD Protein Localized Primarily in the Nucleus.

LNCaP sublines stably expressing CHES1 were established in order to study the effects of its enforced expression in this CaP model. The full-length *CHES1* cDNA was cloned and expressed as an amino-terminal HA- or carboxyl-terminal *mycHis* epitope-tagged protein. Since CHES1 is a transcription factor, its cellular localization was of particular interest; therefore, as an initial step in its biochemical characterization, nuclear and cytosolic fractions of LNCaP-*HA-CHES1* and LNCaP-*CHES1-mycHis* cells were immunoblotted for CHES1 expression. In both cases, CHES1 migrated with an apparent molecular mass of ~56 kD and its expression was predominantly nuclear **(Fig. 5A)**. This contrasted its family member FKHRL1, which exhibits phosphorylation-regulated localization, being primarily cytosolic in the presence of PI3K/Akt pathway activation, but rapidly shifting to the nucleus upon its dephosphorylation during PI3K inhibition with wortmannin **(Fig. 5B)**.

Enforced Expression of CHES1 in LNCaP Cells Induces Phenotypic and Biochemical Features Characteristic of Androgen Withdrawal.

Next, the influence of CHES1 upon LNCaP biology was evaluated. Enforced CHES1 expression resulted in morphological changes indicative of NED characterized by rounded cell bodies and the extension of short neuritic processes, similar to that observed for LNCaP-*vector* cells subjected to androgen withdrawal **(Fig. 6A**, *compare LNCaP-CHES1 panels to LNCaP-vector in CDT-FBS***)**. In contrast to LNCaP-*vector* cells, the NE phenotype of LNCaP-*CHES1* cells did not require androgen deprivation and was evident even in the presence of androgen (*see FBS and CDT-FBS+DHT panels*). Constitutive expression of CHES1 in LNCaP also resulted in a significant decrease in proliferation in the presence of androgen, as LNCaP-*CHES1* cultures exhibited total cell numbers 42% less than that of LNCaP-*vector* cells after five days **(Fig. 6B)**. In the absence of androgen, both cell lines displayed markedly attenuated growth. Having observed that stable CHES1 expression induced growth inhibition and NE differentiation, we wanted to determine if it imparted biochemical features of androgen deprivation. To that end, the levels of selected proteins characteristic of androgen ablation **(Fig. 1A)** were compared in LNCaP-*CHES1* cells and in LNCaP-*vector* cells subjected to androgen withdrawal for three and five days. As shown in **Figure 6C**, LNCaP-*CHES1* possessed a very similar profile to that of androgen-deprived LNCaP cells. AR levels were markedly down-regulated, and only detectable upon longer exposures (*data not shown*). NSE expression was significantly increased, consistent with the NE phenotype of LNCaP-*CHES1*. In addition, serine473-phosphoryated Akt and total Akt levels were greatly elevated. Taken together, these data demonstrate that stable expression of CHES1 can recapitulate several events triggered by androgen ablation.

DISCUSSION

The primary goal of these experiments was to identify and characterize novel androgen withdrawal-regulated genes as they are potentially critical in mediating the biological effects of this treatment and in antagonizing apoptosis. The results of our microarray analysis of LNCaP cells subjected to androgen deprivation generated a transcriptional program composed of 159 differentially expressed genes (*i.e.*, $\geq$2.5-fold) signifying the occurrence of androgen ablation and NED. Although the design of our study was opposite to that of previously described studies involving stimulation with androgen (29, 31), it yielded consistent results in terms of the number (*i.e.*, 146 genes displaying $\geq$3.0-fold changes) (29) and identities of androgen-responsive genes **(Supplementary Tables 1 and 2; Fig. 2, A and B)**. In addition, elevated expression of CD24 antigen and α-tubulin (TUBA3) was in accord with NED and similar to that observed in LuCaP 49 prostatic small-cell, NE carcinoma xenografts (32).

CHES1 displayed an expression pattern typical of an androgen-repressed gene **(Supplementary Tables 1 and 2; Fig. 2, A and B)**. Although we do not have experimental evidence, the results of *in silico* analysis of the *CHES1* genomic locus suggest that it would not be controlled by AR binding to the proximal promoter region or even an upstream enhancer in the 5´-UTR since the nearest putative ARE is located at position -27,386 (*i.e.*, relative to the start site). In contrast, 27 putative ARE/ARR's were found in introns 1, 2, 3, and 5 **(Fig. 3B)**. Similarly, AREs contained in enhancer elements responsible for the tissue-specific expression and androgen repression of the prostate-specific membrane antigen/folate hydrolase 1 (33) and AR (34) genes have not been found in their promoters or 5´-flanking regions, but rather reside in the third intron 12-kbp downstream from the start site or in exons 4/5, respectively. Whether or not *CHES1* is a direct or secondary AR target gene remains to be determined.

CHES1/FOXN3 represented an exciting androgen withdrawal-induced gene since it had been discovered as a putative human checkpoint gene by virtue of its ability to suppress the lethality of multiple checkpoint-deficient yeast strains caused by genotoxic stresses, such as ultraviolet and ionizing radiation and methyl methanesulfonate (22). The full-length *CHES1* transcript encodes a forkhead/winged-helix transcription factor most similar to the FOXN subfamily members WHN/FOXN1 and HTLF/FOXN2 (22). Its predicted molecular mass of 54-kD for the 491-amino acid open reading frame was confirmed in this study **(Fig. 5A)**. However, the ability to increase survival is attributed to its capacity to restore the DNA damage-induced delay of G2/M progression through directly interacting with the corepressor Sin3 and consequently inhibiting the Sin3/Rpd3 HDAC complex (35). HDAC can participate in the recognition of damaged DNA through the formation of a complex with Rad9 (36). Taken together, this suggests that in addition to global effects upon gene transcription, modulation of HDAC activity by CHES1 could also regulate the accessibility of damaged DNA to Rad repair complexes.

The results of our experiments suggest that *CHES1* contributes to the regulation of cell growth and apoptosis of human prostate cancer cells. RNAi-mediated silencing of *CHES1* expression caused a moderate reduction in both the basal androgen-dependent growth of LNCaP cells as well as growth in androgen-deprived medium **(Fig. 4A)**. It is worth noting that the magnitude of this effect might be underestimated since the siRNA transfection efficiency was approximately 80%. Our findings are in accord with previous reports that demonstrated forkhead transcription factors in humans (FKHRL1) (37) and yeast (*Fkh1*, *Fkh2*) (38) promoted cell cycle progression via their action on G2/M promoters, such as *cyclin B* and *polo-like kinase*. The anti-apoptotic role for CHES1 during androgen withdrawal was brought to light by the ability of CHES1 siRNAs to convert combined growth inhibition and NED to an apoptotic response **(Fig. 4B)**. This is consistent with its previously described survival properties in response to genotoxic insults (22) as well as its ability to functionally replace *MEC1* (yeast homolog of

ATM/ATR) as an essential factor for growth of yeast even in the absence of DNA damage. Additionally, RNAi of *CHES1-like* induced marked apoptosis of *Drosophila melanogaster* macrophage-like S2 cells (39). Due to the toxicity of *CHES1* silencing during androgen withdrawal, these experiments were inconclusive in terms of defining a role for *CHES1* in NED. We observed that *CHES1* expression was repressed in response to p53 activation by IR **(Fig. 2D)**. This might be explained by the presence of a putative p53 half-site in the *CHES1* promoter (*data not shown*). Although it was not cytotoxic, IR has been reported to sensitize LNCaP cells to TRAIL-mediated apoptosis through the coordinated up-regulation of death receptor 5 and modulators of the intrinsic apoptosis pathway, Bax, and Bak (40). Similarly, the anti-cancer agents mitomycin C and doxorubicin were shown to increase apoptosis susceptibility via a p53-dependent induction of the CD95/APO-1/Fas death receptor (41) and cytotoxic doses of both of these agents repressed the expression of *CHES1* in HepG2 human hepatocellular carcinoma cells (42). Consistent with p53-mediated *CHES1* repression as a pro-apoptotic mechanism, it is noteworthy that long-term androgen withdrawal was accompanied by diminished p53 protein levels (*data not shown*).

The growth rate of LNCaP-*CHES1* was retarded by approximately 42% compared to that of vector-transfected cells **(Fig. 6B)**. While this result was paradoxical when considered together with the findings from our RNAi experiments **(Fig. 4A)**, it might be explained by the fact that enforced expression of *CHES1* at elevated levels mediated a cellular response of differentiation (*discussed below*) and growth inhibition. In the LNCaP model, there are precedents for one stimulus mediating opposite responses. While androgen is a potent mitogen at physiological concentrations, growth repression and apoptosis occur at supraphysiological doses (43). In fact, AI LNCaP sublines established by long-term cultivation in hormone-depleted medium consistently exhibit a paradoxical growth inhibition in response to androgenic stimulation (16, 44). Along the same line, Akt-mediated phosphorylation of the AR has differential effects on its activity in that it can suppress AR activity at low passage numbers or enhance it at higher ones (45).

Stable expression of *CHES1* in LNCaP partially recapitulated the biological and molecular responses to androgen withdrawal. The acquisition of a NE phenotype of LNCaP-*CHES1* cells was evidenced both morphologically and biochemically **(Fig. 6, A and C)** and was very stable in that it was not altered by hormonal manipulation **(Fig. 6A)** and has remained unchanged to date. The ability of *CHES1*/*FOXN3* to induce differentiation was not completely unexpected as *Whn*/*FOXN1*, whose mutation gives rise to the *nude* phenotype in mice and rats, has an obligatory function in terminal differentiation of cutaneous and thymic epithelial cells (46). Since *CHES1* overexpression did not induce NED identical in appearance to that of androgen withdrawal, we interpret this to indicate that it acts in concert with other components of the differentiation program and potentially at a later stage, based upon the kinetics of its expression. As an example, increased expression of receptor-type protein-tyrosine phosphatase alpha during androgen deprivation has also been implicated as a vital component of NED via MAPK/ERK signaling (47). Interestingly, the NED phenotype in LNCaP varies with the stimulus, such as interleukin-6 (48) and agents which elevate intracellular cAMP levels and activate protein kinase A (49). It was surprising that *CHES1* could promote NED in the presence of androgen, but the markedly diminished levels of AR in LNCaP-*CHES1* cells **(Fig. 6C)** could explain this since it has been reported that silencing of *AR* expression was sufficient to induce NED (8). While markedly elevated Akt activation **(Fig. 6C)** was an additional characteristic of androgen withdrawal, it also poses as a mediator of AR protein diminution via triggering ubiquitin-mediated degradation by its phosphorylation of the AR (50). Interestingly, it has recently been reported that Whn/FOXN1 induced the expression of Akt and chromogranin A during terminal differentiation of primary human keratinocytes (46).

The identification and role of human *CHES1* as a critical mediator of G2/M and mitotic spindle checkpoints, basal cell growth, and enhancement of survival in response to genotoxic stresses have been elegantly elucidated in the yeast system (22, 35). In this manuscript, we extended these findings to characterize its role in prostate cancer biology. Our results demonstrated that it is regulated in an androgen- and p53-repressed manner and was capable of recapitulating phenotypic and molecular features of androgen withdrawal. CHES1 also contributes to the androgen-dependent growth of LNCaP cells and confers resistance to apoptosis during androgen withdrawal. Since *CHES1* silencing resulted in apoptosis specifically during androgen ablation, the adjuvant use of agents that antagonize its expression or function might be effective in delaying the development of hormone refractory disease.

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ABBREVIATIONS

AI, androgen-independent; AD, androgen-depleted; AR, androgen receptor; ARE, androgen response element; ARR, androgen response region; AW, androgen withdrawal; CaP, carcinoma of the prostate; CDT-FBS, charcoal/dextran-treated-FBS; *CHES1*, checkpoint suppressor 1; DBD, DNA binding domain; FBS, fetal bovine serum; GAPD, glyceraldehyde 3-dehydrogenase; HDAC, histone deacetylase; IGF-1, insulin-like growth factor-1; IR, ionizing radiation; NE, neuroendocrine; NED, neuroendocrine differentiation; NSE, neuron-specific enolase; PI3K, phosphatidylinositol 3-kinase; PSA, prostate-specific antigen; RNAi, RNA interference; SAGE, serial analysis of gene expression; siRNA, small interfering RNA.

FIGURE LEGENDS

Figure 1. Androgen ablation of LNCaP induces marked modulation of AR-regulated protein levels and neuroendocrine differentiation. ***(A)*** LNCaP cells were left untreated in the presence of DHT (1 nM) or were subjected to conditions of androgen ablation for a duration of 1-45 days by culturing in RPMI 1640 medium (without phenol red) supplemented with CDT-FBS. Immunoblot analysis of NP-40 lysates was performed for the determination of AR, NSE, phospho(Ser473)-Akt, and total Akt levels. Migration of molecular weight standards is indicated. ***(B)*** Morphology of LNCaP cells cultured for five days in the presence or absence of androgen was evaluated by phase contrast microscopy at 200X magnification. Androgen withdrawal induced the appearance of characteristics typical of NED, such as extension of neuritic processes and rounded cell bodies.

Figure 2. Microarray analysis of LNCaP cells subjected to androgen withdrawal yields a distinctive expression profile characterized by marked up-regulation of *CHES1*. ***(A)*** Total RNA was prepared from LNCaP cells cultured in the presence of androgen (*+DHT*) and subjected to androgen withdrawal (*-DHT*) for 0.5, 6, 24, and 96 hours. Global gene expression profiling was performed with Affymetrix HG-U95Av2 GeneChip oligonucleotide arrays as described in *Materials and Methods*. Comparison analysis and hierarchical clustering (dChip) were used to identify transcripts that exhibited $\geq$2.5-fold differential expression at the 96-hr time point and then to group those with similar expression patterns, respectively. The resulting cluster diagram depicts two major clusters of *androgen withdrawal (AW)-repressed* and *AW-induced* genes. The mean expression value for each gene throughout the time course was determined and the magnitude of increased or decreased expression at each time point relative to the mean is depicted by increasingly darker shades of red or blue, respectively. As shown in the right panel, *CHES1* segregated with the "AW-induced" gene cluster containing 78 transcripts and displayed strong induction by 24 hours. ***(B)*** *CHES1* is up-regulated during androgen withdrawal and exhibits kinetics comparable to other "androgen-repressed" genes. Fold changes in expression (relative to LNCaP cultured in the presence of androgen; *+DHT*, *empty bars*) for *CHES1* and the indicated androgen-regulated genes at each time point were determined by comparison analysis of the data obtained from the microarray experiment described in ***Fig. 2A***. Results are graphically represented for each gene following 0.5 (*diagonally-hatched bars*), 6 (*horizontally-lined bars*), 24 (*checkered bars*), and 96 (*solid bars*) of androgen withdrawal. ***(C)*** AW-induced CHES1 expression was validated by standard RT-PCR analysis of total RNA prepared from LNCaP cells at the indicated times after the beginning of androgen deprivation. Expression in androgen-independent LNCaP-cds cell lines (clones 1 and 2) was also evaluated. α3-tubulin levels were monitored as a positive control for AW-induced gene expression and glyceraldehyde 3-phosphate dehydrogenase (GAPD) as a loading control and for normalization. ***(D)*** *CHES1* expression is suppressed by p53 activation. LNCaP cells

(2.5×10^6) were seeded in 10-cm dishes and subjected to ionizing radiation (10 Gy) for the indicated times or left untreated for 24 hours (*Con*). Total RNA was isolated and analyzed by RT-PCR for the expression of CHES1, $p21^{Cip1/Waf1}$, and β-actin.

Figure 3. *CHES1* genomic locus and mapping of potential androgen-response elements. ***(A)*** Structure of the *CHES1* gene and polypeptide. The *CHES1* genomic locus maps to chromosome 14q24.3-q31 (22) and encodes six exons. These were identified by BLAST analysis of human genome sequence (NCBI) with the full-length 1,473-bp *CHES1* cDNA sequence (NM_005197[gi:4885136]) and are shown positioned along contig NT_026437.10 containing the CHES1 locus. The full-length isoform is referred to as CHES1α and encodes a 490-amino acid polypeptide. CHES1β, an alternatively spliced variant lacking exon 4, is also present in LNCaP. The structures of the mouse (*Mus musculus*) orthologs, *Mm CHES1* and *Mm similar to CHES1*, are shown for comparison. The conserved forkhead/winged helix domain is shaded in gray. ***(B)*** Putative sites of AR binding in the *CHES1* gene. The 312.6-kbp contig sequence containing the entire *CHES1* coding region and 5´- and 3´- untranslated regions was screened for the presence of AREs and ARRs and are depicted as hash marks. The consensus sequence used for screening is shown below the schematic.

Figure 4. RNAi-mediated CHES1 silencing induces growth inhibition and apoptosis of LNCaP cells. ***(A)*** LNCaP cells were seeded in 96-well plates (1×10^4 cells/well) in the presence (+*DHT*) or absence (-*DHT*) of androgen and transfected with one of three CHES1-specific siRNA duplexes (100 nM) as described in *Materials and Methods*. As a control for any non-specific effects of the transfection reagent and/or siRNA duplex, cells were also transfected with an siRNA that targeted the firefly luciferase gene. Following a three-day incubation, proliferation was determined by MTS assay. The mean absorbance (A490 nm) of quadruplicate wells for each CHES1 siRNA-treated group was used to determine fractional growth relative to the control siRNA treated group (*empty bars*). Results are expressed as percentage growth inhibition for each CHES1 siRNA (*diagonally-hatched, checkered, and solid bars*) relative to the control ± S.D. ***(B)*** LNCaP cells were seeded in 6-well plates under the growth conditions described in *panel A* and transfected with CHES1 (*CHES1-Ri-1*) or luciferase (*Control*) siRNAs. Cell morphology was examined by phase contrast microscopy under 200x magnification. In the absence of androgen (-*DHT*), CHES1 RNAi induced characteristic features of apoptosis. ***(C)*** Total RNA was isolated from samples treated identically as in *panel B* as well as from cells mock-transfected with siRNA universal buffer (*UB*). RT-PCR analysis of CHES1 and AR expression was performed and validated the effectiveness and specificity of the CHES1 siRNA (*Ri*) treatment.

Figure 5. CHES1 is constitutively localized to the nucleus. ***(A)*** Nuclear and cytosolic extracts were prepared from LNCaP sublines stably transfected with *myc*His or HA epitope-tagged CHES1 expression constructs and subjected to immunoblot analysis to examine CHES1 cellular localization. LNCaP cells transfected with empty vector (*Vector*) were included as a negative control. Tagged CHES1 was detected by probing with monoclonal anti-HA (*upper panel*) or anti-*myc* (*lower panel*) antibodies. CHES1 expression was detected primarily in the nuclear fraction. Migration of molecular weight standards are indicated on the left-hand side of the gel. The anti-*myc* antibody also detected a non-specific protein migrating at ~49.8 kD. ***(B)*** Phosphorylation-regulated FKHRL1/FOXO3A cellular localization was demonstrated by immunoblot analysis of nuclear and cytosolic extracts from LNCaP cells treated with the PI3K inhibitor wortmannin (100 nM) for 1 and 3 hours. The blot was developed with polyclonal anti-phospho (Thr32)-FKHRL1 and anti-FKHRL1 antibodies.

Figure 6. Enforced expression of CHES1 recapitulates phenotypic and molecular responses of androgen withdrawal. ***(A)*** LNCaP-*CHES1* cells have a neuroendocrine morphology. LNCaP-*CHES1* and LNCaP-*vector* cell lines were cultured in 10-cm dishes for 5

days in medium containing the indicated serum and/or androgen supplements. Digital images were acquired using phase contrast microscopy at 200x magnification. Under all conditions, LNCaP-*CHES1* cells have rounded cell bodies and neuritic processes. ***(B)*** Enforced CHES1 expression retards androgen-stimulated proliferation. LNCaP-*vector* (■, □) and LNCaP-*CHES1* (●, ○) cells were seeded in 10-cm dishes at a density of 3 x 10^5 cells/dish. After attachment, the medium was changed to assess growth in the presence (*filled symbols*) or absence (*open symbols*) of DHT (1 nM). Cell numbers were determined for each sample in triplicate by cell counts with a Neubauer hemocytometer on days 2, 3, and 4. Results are expressed as mean total number of cells per dish ± S.D. ***(C)*** LNCaP-*CHES1* cells display biochemical features typical of LNCaP cells subjected to androgen withdrawal. NP-40 lysates were prepared from LNCaP-*vector* cells cultured in androgen-containing (*FBS*) or depleted (*CDT-FBS*) medium and from LNCaP-*CHES1* cells cultured in the presence of androgen. Immunoblot analysis was performed to confirm HA-CHES1 expression and to compare levels of AR, phospho(Ser473)-Akt, total Akt, and NSE. GAPDH was used as a loading control and molecular weight markers are indicated.

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mTOR Integrates Androgen Receptor and PI3K-Akt Signaling During Androgen Withdrawal

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ABSTRACT

A network of anti-apoptotic mechanisms promotes the survival of prostate cancer (CaP) cells during androgen ablation and facilitates the development and persistence of castration-recurrent prostate cancer (CRPC). The goal of this work was to better define these with the operating hypothesis that the efficacy of androgen ablation can be enhanced with adjuvant targeting of one or more survival pathways. In the androgen-sensitive LNCaP model, diminished androgen receptor (AR) signaling represents the pivotal response to androgen withdrawal (AW) and mediates survival via hyperactivation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and elevated Bcl-2 levels. Our results demonstrate that AR-dependent mTOR complex 1 (mTORC1; rapamycin-sensitive) negatively regulates this response and that disruption of the AR-mTORC1-Akt signaling axis represents a critical molecular event in the transition to CRPC since PI3K-Akt levels are markedly elevated in androgen-independent (AI) LNCaP-cds sublines despite restoration of AR signaling. Importantly, the hyperdependence of LNCaP on PI3K for survival during AW was demonstrated by the ability of the specific inhibitor LY294002 to induce marked apoptosis. Interestingly, clinically-relevant agents, such as adriamycin (doxorubicin), induced LNCaP apoptosis by performing dual actions as mimetics of androgen ablation (AR degradation) and signal transduction inhibitors (mTORC1 inhibition). In contrast, LNCaP-cds cell lines were remarkably resistant to these treatments, despite p53 induction and AR degradation. Further studies revealed that PI3K-Akt signaling was refractory to LY294002 and implicated Bcl-2 overexpression as the downstream mechanism conferring pan-resistance to multiple apoptotic stimuli. This notion was confirmed by the ability of the Bcl-2 inhibitor HA14-1 to sensitize LNCaP-cds cells to adriamycin. In summary, our results suggest that *1)* the progression of CaP to CRPC is mediated via the transient de-repression and eventual loss of AR-mediated negative regulation of anti-apoptotic mechanisms and *2)* the success of targeting these mechanisms during androgen ablation is influenced by cellular context.

INTRODUCTION

Androgen ablative therapy exploits the strongly androgen-dependent character of prostate cancer (CaP) and is the first-line therapy for the treatment of metastatic disease. Although this dependably mediates regression, it is only palliative since recurrence typically occurs in the form of castration-resistant prostate cancer (CRPC) in 2-3 years **(1)** and is commonly referred to as androgen-independent (AI) CaP. A significant obstacle to the successful treatment of CaP is that in contrast to terminally-differentiated, non-malignant prostate epithelial cells, androgen deprivation results in apoptosis of only a sub-population of tumor cells. Although AR signaling and protein levels are substantially diminished, androgen withdrawal induces several anti-apoptotic mechanisms that compensate for the loss of the potent survival signal elicited by androgen. These include phosphatidylinositol 3-kinase (PI3-K)-Akt/PKB signaling **(2)**, Bcl-2 **(3)**, IL-17R **(4)**, and clusterin (CLU)/testosterone-repressed prostate message-2 **(5)**. Accordingly, their individual roles are validated by the ability to mediate cell death with small molecule or antisense inhibitors of these and have provided a basis for their clinical application **(6)**. However, the mechanism(s) through which these responses are coordinated are currently ill-defined. However, there is a possibility for mechanistic connection based upon the fact that Akt phosphorylation of AR leads to its ubiquitin-mediated degradation and that mTOR suppresses AR. At the same time mTOR

Although CRPC is generally defined by tumor recurrence and biochemical failure during androgen ablation, it is actually a heterogeneous disease that is further complicated by metastasis and that exhibits a multi- or pan-resistant phenotype, being refractory to anti-androgens and conventional radiation and chemotherapy. As a result, there are presently no broadly effective treatments for its clinical management. One of the most prominent features of the transition to castration resistance is the reinstatement of AR signaling, despite androgen depletion and the presence of an anti-androgen. This is characterized by restored AR protein expression and an incomplete reinstatement of the AR transcription program in that the expression of a number of androgen-regulated genes does not return to the level prior to androgen deprivation **(7)**. Notably, the *AR* gene itself is one of these genes and while its expression is elevated upon androgen withdrawal, its persistent overexpression is a consistent feature of recurrent tumors in experimental models and clinically **(8)**. Similarly, the mechanisms discussed above are refractory to AR signaling and remain elevated

In this manuscript, we demonstrate that mTORC1 is a key target of androgen action and integrates androgen withdrawal-induced survival mechanisms and AR down-regulation. Conversely, functional dissociation of the AR-mTORC1-Akt signaling axis and PI3K deregulation are associated with the transition to CRPC and contribute to persistent Akt hyperactivation, Bcl-2 overexpression, and restoration of AR expression. These aberrations result in a pan-resistant phenotype that is refractory to PI3K inhibitors, but can be sensitized to chemotherapy by antagonism of Bcl-2 function. In addition, the results demonstrate that mTORC1 has both androgen-dependent and –independent components, but requires androgen for full activity in the context of androgen-dependent CaP cells.

EXPRIMENTAL PROCEDURES

Cell Lines and Culture.

LNCaP and 22R*v1* prostate adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 2 mM L-glutamine, and 100 U/ml penicillin-100 μg/ml streptomycin at 37°C in a humidified environment of 5% CO_2 in air. To simulate conditions of androgen ablation, cultures were shifted into RPMI 1640 medium (without phenol red) supplemented with 10% charcoal/dextran-treated fetal bovine serum (CDT-FBS; Omega Scientific). LNCaP-cds were generated by long-term cultivation in the absence of androgen maintained under conditions of chronic androgen deprivation as described previously **(16)**. The LY294002-resistant LNCaP subline (LNCaP-LY) was generated and maintained by continuously culturing LNCaP cells in the presence of 20 μM LY294002.

Reagents.

R1881 was purchased from Sigma-Aldrich (St. Louis, MO). LY294002, rapamycin, PI-103, and HA14-1 were purchased from Calbiochem/EMD Chemicals, Inc. (Gibbstown, NJ). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was purchased from Roche Applied Science (Indianapolis, IN). Mouse monoclonal antibody against the androgen receptor (clone AR 441, Ab-1) was purchased from Lab Vision Corporation, (Fremont, CA) and rabbit polyclonal anti-AR (PG-21) was purchased from Upstate Biotechnology/Millipore (Temecula, CA). Rabbit anti-phospho-p70S6K(Thr389), anti-p70 S6K, anti-phospho(Ser473)-Akt, anti-Akt, anti-phospho-Phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204), and anti-Bcl-2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-HA (HA.11, clone 16B12) was purchased from Covance Research Products (Berkeley, CA). The mouse monoclonal antibody against GAPDH (clone 6C5) was obtained from Chemicon International (Temecula, CA).

Immunoblot Analysis.

Immunoblot analysis was performed according to standard protocols as previously described **(17)**. Briefly, cell lysates were prepared in NP-40 lysis or radio-immunoprecipitation assay (RIPA) buffer. Proteins were quantitated using the BCA protein assay (Pierce, Thermo Fisher Scientific, Rockford, IL). Twenty-five micrograms protein were resolved by SDS-PAGE and then transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA). The membranes were blocked in 5% BSA/TBST or 5% non-fat dry milk/TBST and then incubated with the appropriate primary and HRP-linked secondary antibodies. Subsequently, membranes were developed with ECL Plus enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) and digitally imaged.

Apoptosis Assays.

DAPI Staining. Nuclear morphological changes characteristic of apoptosis were visualized by staining cells with DAPI. Cells were treated on chamber slides and fixed in 4% paraformaldehyde/1X PBS for 30 minutes at room temperature. The slides were then washed twice with 100% ethanol, once with 70% ethanol, once with 1X PBS, and then incubated in DAPI staining solution (0.2 μg/ml DAPI/0.1% Triton X-100/2% paraformaldehyde/1X PBS) for 30 minutes at 4°C and protected from light. Samples were examined with an Olympus BX61 fluorescence microscope using a DAPI filter cube (Excitation wavelength: 350±50 nm, Dichroic:

400. Emission wavelenth: 460±50 nm). Images were acquired and processed using SlideBook 4.1 digital microscopy software (Olympus, Center Valley, PA). Percentage apoptosis was determined by counting the number of apoptotic nuclei in random fields of a total of 300 cells.

Annexin V Binding Assays. In order to quantitate apoptosis-associated phosphatidylserine externalization, Annexin V binding assays were performed according to a previously described protocol **(18)**. Cells (1 x 10^6) were seeded into 60-mm tissue culture dishes and treated with vehicle control (DMSO) or PI3K inhibitors for 24 hours. The entire culture was harvested for analysis by recovering any floating cells in the medium and trypsinization of the remaining adherent cells. The cells were collected by centrifugation and washed once with serum-containing growth medium and then with HEPES binding buffer (10 mM Hepes-NaOH, pH 7.4/150 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1.8 mM $CaC1_2$). The cells were then incubated at room temperature with Annexin V-FITC (1 μg/ml; Clontech Laboratories, Inc., Mountain View, CA) for 10 minutes in the dark and analyzed by flow cytometry.

Retrovirus Production, Infection, and Establishment of Stable Cell Lines

Retroviruses were produced according to standard protocols **(19,20)**. Briefly, pBABE-PuroL and pBABE-FKHR expression constructs (2 μg) were transfected into LinX-A amphotropic packaging cells (Open Biosystems, Huntsville, AL) using FuGENE 6 transfection reagent (Roche Applied Science). Cultures were incubated at 32°C for 72 hours and the virus-containing supernatant was collected, centrifuged at 3,000 *x g* for 15 minutes at 4°C, and filtered through a 0.45-μm surfactant-free cellulose acetate membrane (Corning, Inc.). LNCaP and LNCaP-cds3 cells (40-50% confluent) were infected with supernatants diluted 1:1 in complete RPMI 1640 medium supplemented with polybrene (4 μg/mL) and incubated for 24 hours at 32°C. Subsequently, stable clones were derived and maintained by selection in appropriate growth medium containing puromycin (1 μg/mL) at 37°C.

m^7GTP Cap Binding Assays

These were performed with slight modification to a previously described protocol **(21,22)**. Cells extracts were prepared in a modified cap binding assay lysis buffer (CLB; 40 mM HEPES, pH 7.2/10 mM $MgCl_2$/1 mM EDTA, pH 8.0/0.5% NP-40/0.1% Brij 35/1 mM sodium orthovanadate, 15 mM β-glycerophosphate) supplemented with protease inhibitors (2 μg/ml each of aprotinin, leupeptin, pepstatin A; 0.5 mM AEBSF). Extracts (600 μg protein) were then incubated with 7-methyl GTP Sepharose 4B beads (GE Healthcare, Piscataway, NJ) at 4°C for 2.5 hours. The beads were washed three times with CLB and bound proteins were released by resuspension in 2X Laemmli sample buffer containing 2% β-ME and heating at 95°C. Eluted proteins were resolved by SDS-PAGE and analyzed by immunoblot analysis.

RESULTS

Anti-apoptotic mechanisms are induced by androgen withdrawal and persist in androgen-independent cells.

Androgen withdrawal (AW) induces growth arrest and neuroendocrine differentiation (NED) of LNCaP human prostate adenocarcinoma cells. Although androgen receptor (AR) signaling and expression are greatly diminished, these cells exhibit a persistent capacity to survive as demonstrated by the absence of significant levels of apoptosis. In the absence of androgen, the phosphatidylinositol 3-kinase (PI3-K)-Akt/PKB pathway activity is elevated and serves as a dominant mediator of survival **(2,15)**. As a model system to investigate prostate cancer survival mechanisms operating during the transition from androgen ablation to the development of androgen independence, we utilized *1)* androgen-sensitive LNCaP cells subjected to short- and medium-term androgen withdrawal (AW) and *2)* androgen-independent LNCaP-cds sublines derived by long-term culture in the absence of androgen **(16)**, respectively. Immunoblot analysis demonstrated that AW mediated rapid and persistent elevations in the levels of Ser473-phosphorylated Akt and Bcl-2 coincident with a reduction in AR expression beginning after one day of AW and persisting for the 45-day duration of the experiment **(Fig. 1A)**. Similarly, AI LNCaP-cds clones that are maintained continuously in the absence of androgen exhibited marked Akt hyperactivation and Bcl-2 overexpression **(Fig. 1B)**. In order to evaluate the dependency of parental and AI LNCaP cells upon PI3K-Akt signaling for survival during androgen deprivation, both cell types were treated with PI3K inhibitors followed by quantitation of apoptotic cells with DAPI staining. As shown in **Fig. 1C**, LY294002 and wortmannin treatments induced marked apoptosis of LNCaP cells specifically in the absence of androgen (38% and 78%, respectively) as evidenced by characteristic changes in nuclear morphology such as karryorhexis and pyknosis. In contrast, AI LNCaP-cds cells were refractory to PI3K inhibitors; apoptotic morphology was not evident by DAPI staining (*data not shown*) and the more sensitive Annexin V binding assay detected only marginal amounts of apoptosis (range: 0.5–3%) **(Fig. 1D)**. In summary, the data demonstrate that while PI3K-Akt signaling is hyperactivated as a response to AW and is conserved after the transition to androgen independence, PI3K inhibitors are only effective in mediating apoptosis of androgen-sensitive parental LNCaP cells, but not AI LNCaP-cds sublines.

Androgen independence is associated with the acquisition of multiple survival signaling pathway aberrations.

In order to gain insight into the basis for resistance of LNCaP-cds cells to PI3K inhibitors, we first examined their effects upon Akt activation. As expected, S473 phosphorylation of Akt in LNCaP was almost completely abolished by treatment with LY294002 and wortmannin for 24 hours **(Fig. 2A)**. In contrast, phospho-Akt(S473) levels in identically-treated LNCaP-cds cells were only partially reduced and remained either equivalent to or higher than that found in untreated LNCaP **(Fig. 2A)**. As a complimentary approach to understanding the basis of resistance to PI3K inhibitors, a LY294002-resistant subline (LNCaP-LY) was generated by continuous culture of LNCaP in the presence of LY294002 **(Fig. 2B)**. Interestingly, these cells had relatively high levels of phospho-Akt(S473) and elevated expression of AR. Taken together, these data demonstrate that the development of resistance to androgen ablation is associated with acquisition of abnormalities in the regulation of PI3K signaling, which might contribute to the AI phenotype via overexpression of AR and Bcl-2.

Since the maintenance of Akt activity could explain the observed apoptosis resistance, we wanted to determine if apoptosis could in fact be induced if PI3K-Akt signaling was successfully inhibited, or bypassed, and the function of pro-apoptotic Akt target molecules was restored. To this end, expression of a constitutively-active FOXO1/FKHR mutant (*i.e.*, FKHR/AAA Akt phosphorylation mutant) was enforced in LNCaP and LNCaP-cds3 cells by retroviral gene transfer. Infection with retroviruses encoding wild-type, DNA-binding domain mutant (H215R), and combination AAA+H215R FKHRs were used as controls and for comparison. Subsequently, the infected cells were selected by puromycin and expression of the various FKHR proteins was confirmed by immunoblot analysis with anti-HA antibody **(Fig. 2C)**. While expression of FKHR/AAA was toxic to parental LNCaP cells, which could not be propagated in the presence of puromycin, a stable cell line could be established from LNCaP-cds2 (LNCaPcds3-FKHR/AAA). Growth was monitored with cell counts and demonstrated that FKHR did not influence growth substantially. In conclusion, reinstatement of one pro-apoptotic signal downstream of PI3K is insufficient to mediate apoptosis in AI LNCaP-cds2 cells.

In order to determine if apoptosis of AI cells could potentially be mediated via a different pathway, cells were treated with doxorubicin in order to trigger p53 activation. Although this markedly induced p53 levels in both LNCaP parental and cds2 lines, apoptosis, as evidenced by PARP cleavage, was only induced in the former. Taken together with the results from the experiments described above, this demonstrates that the LNCaP-cds series of sublines possess a pan-resistant phenotype. We reasoned that the overexpression of Bcl-2 **(Figs. 1B, 2A)** was the basis for this and hypothesized that apoptosis resistance could be overcome by antagonizing its function with the small molecule, non-peptidic Bcl-2 ligand HA14-1. Cells were treated for 24 hours with doxorubicin and HA14-1 (10, 25, and 40 μM) alone or in combination and then analyzed for the occurrence of apoptosis. As a single agent, HA14-1 did not induce apoptotic morphological changes or detectable PARP cleavage **(Figs. 2D)**. While doxorubicin induced a low level of PARP cleavage and release of cytochrome *c*, these events were increased 2-3-fold in combination with HA14-1 and accompanied by the appearance of apoptotic cells **(Figs. 2E)**. Under conditions of apoptosis, phospho-Akt(S473) levels were unchanged, or slightly elevated, compared to that of untreated cells. In summary, AI LNCaP-cds cells can be re-sensitized to apoptosis by inhibition of Bcl-2 function even in the presence of strong PI3K-Akt signaling.

Mammalian target of rapamycin (mTOR) acts as a critical sensor of androgen signaling and integrates androgen withdrawal-induced PI3K-Akt hyperactivation and AR down-regulation.

As discussed above, androgen withdrawal triggers several biochemical events including *1)* AR down-regulation, *2)* hyperactivation of the PI3K-Akt pathway, and *3)* Bcl-2 overexpression. Although androgen represents the pivotal mediator of these events, the mechanisms underlying their androgenic regulation and subsequent dissociation remain ill-defined. The persistent hyperactivation of Akt in response to AW represents a potent survival signal. However, the mechanism connecting diminished AR signaling to elevated Akt phosphorylation is currently ill-defined. Based upon its position in signaling pathways upstream or downstream of AR and PI3K-Akt, we hypothesized that mTOR might function in the regulation of one or more of these events.

To investigate this, LNCaP cells were cultured in the presence of synthetic androgen *(+R1881*) or subjected to androgen deprivation for 2, 5, and 7 days. During the incubation, cells

were also treated with the PI3K inhibitor LY294002 (lanes labeled "L") or the mTOR inhibitor rapamycin (lanes labeled "R"). In addition, androgen was re-applied after 5 days (*e.g.*, *AW→R1881*). Immunoblot analysis demonstrated that AW mediated a coincident diminution of AR levels and elevation in phospho-Akt(S473) levels over the time course **(Figure 3A,** compare untreated lanes labeled "-"**)**. Activity of mTORC1 was monitored by evaluating the levels of phosphorylation of S6K1 upon Thr389. While S6K1 was strongly phosphorylated in the presence of R1881, phospho-S6K1(T389) levels were markedly reduced (but not completely eliminated) after 2 and 5 days of AW. As expected, S6K1 phosphorylation was completely inhibited by LY294002 and rapamycin treatments. This was accompanied by an increase in AR expression both in the presence of androgen (*+R1881*, lanes L and R) and in its absence (*AW*, lanes L and R). In fact, mTORC1 inhibition completely blocked AW-mediated AR down-regulation and maintained AR expression at a level higher than that in R1881-treated cells. In the presence of androgen, rapamycin also elevated phospho-Akt(S473) to a level equivalent to the hyperactivation reached after androgen deprivation; rapamycin could not further increase phospho-Akt(S473) levels above that achieved by AW alone. Interestingly, although mTORC1-S6K1 remained sensitive to androgen and could potently be reactivated upon its addition, AW-induced PI3K-Akt activation was refractory to repression by subsequent androgen treatment. Time-course experiments further demonstrated that while R1881 induced partial mTORC1 reactivation within 15 minutes, full reinstatement of phospho-S6K1(T389) levels proceeded with slow kinetics and required 24 hours **(Fig. 3B)**. Taken together, the results demonstrate that mTOR plays a central role in controlling AR down-regulation and Akt hyperactivation during AW. Several pieces of data support this. First, complete inhibition of mTORC1 with rapamycin during AW prevented AR down-regulation while rapamycin treatment of LNCaP under androgen-sufficient conditions increased phospho-Akt levels equivalent to that induced by AW. Androgen withdrawal initiates the process by mediating a dramatic, but incomplete, reduction in mTOR activity. While this reduction is apparently sufficient to de-repress Akt and lead to its hyperactivation, the residual mTOR activity potentially drives down AR expression.

Disruption of the AR-mTORC1-Akt signaling axis represents a critical molecular event in the transition to CRPC

We next wanted to determine if the androgenic control of the AR-mTORC1-Akt axis was conserved or altered after the transition to castration recurrence. To this end we compared the androgen response of these components in the androgen-independent cell lines LNCaP-cds3 and 22R*v1* to that of LNCaP. In the absence of androgen, all three cell lines exhibited similar levels of basal S6K1(T389) phosphorylation, but although it was induced in both AI cell lines, the response was attenuated compared to androgen-dependent LNCaP **(Fig. 4A)**. Consistent with these findings, m^7GTP cap binding assays demonstrated that DHT increased the association of phospho-S6 ribosomal protein (S235/6) with the translation initiation complex in LNCaP cells, but this interaction was reduced in the context of LNCaP-cds3 **(Fig. 4B)**. The functional relationship between mTORC1, Akt, and AR was examined in more detail by treatment of LNCaP-cds3 cells with rapamycin or LY294002, as described for LNCaP **(Fig. 3A)**. In contrast to the androgen-responsiveness of S6K1 phosphorylation in LNCaP-cds3 cells, Akt phosphorylation was generally unaffected by androgen exposure or by mTORC1 inhibition with rapamycin and was only partially inhibited by LY294002 **(Fig. 4C)**. As in the case of parental LNCaP, AR expression could be increased slightly by androgen stimulation for one day. However, the response to mTORC1 inhibition was altered in that although AR levels were

elevated by rapamycin in androgen-free medium (*i.e.*, typical culture conditions for LNCaP-cds3), its expression was reduced in the presence of R1881. Taken together, these results demonstrate that although mTORC1 retains androgen-responsiveness in CRPC cell lines, its repressive effects upon AR expression and Akt activation are compromised.

Androgen and polypeptide growth factors activate mTORC1 through independent mechanisms.

mTORC1 is regulated by nutrients (*e.g.*, glucose, amino acids) and growth factors. Based upon the results presented above, it was important to determine *1)* if the residual, androgen-independent mTORC1 activity was derived from serum-derived growth factors and *2)* if androgen could act independently as an activator of mTORC1. One approach we used to address this was to completely inhibit mTORC1 activity and then determine the capacity of serum and androgen to subsequently reinstate its activity. To this end, LNCaP cells were subjected to androgen withdrawal for 3 days, treated with LY294002 for 2 hours, and then washed **(Fig. 5A,** *AW+LY→Wash→***)**. R1881 (10 nM) was added and the kinetics of mTORC1 reactivation was followed. Basal, androgen-independent S6K1 phosphorylation was completely suppressed by PI3-K inhibition **(Fig. 5A,** *AW+LY***)**, but was fully restored within 15 minutes after the removal of the inhibitor **(Fig. 5A,** *AW+LY→Wash→AW-0.25***)**. Androgen-stimulated mTORC1 activity was not apparent until 4 hours after the addition of R1881 and peaked at 24 hours **(Fig. 5A,** *AW+LY→Wash→R1881 lanes***)**. In contrast, phospho-S6K1 levels were unchanged after the same duration in the absence of androgen **(Fig. 5A,** *AW 24***)**. These results demonstrate that serum-derived growth factors can rapidly activate mTORC1 independently of androgen signaling and that androgen enhances this signal, but requires longer term signaling (*i.e.*, hours).

We next wanted to determine in a more defined manner if androgen could activate mTORC1 independently of growth factors, and *vice versa*. To this end, androgen-deprived LNCaP cells were treated with R1881 (1 nM) alone or in combination with epidermal growth factor (EGF) or heregulin-β1 (HRG-β1), and in the presence or absence of serum (10% CDT-FBS). A low, but easily detectable, level of phospho-S6K1(T389) was observed in cells under conditions of androgen-deprivation **(Fig. 5B, +***Serum, lane 1***)** and serum starvation for 3 days **(Fig. 5B, -***Serum, lane 1***)**. Notably, R1881 treatment alone elevated S6K1(T389) phosphorylation levels even in the complete absence of serum **(Fig. 5B, -***Serum, lane 2***)**. On the other hand, short-term treatment with EGF stimulated mTORC1 in an androgen-independent manner; however, maximal EGF-induced phospho-S6K1(T389) levels were achieved in combination with R1881 and appeared to be additive with that derived from androgen stimulation **(Fig. 5B**, *compare EGF "+" and " –" lanes***)**. In contrast, HRG-β1 did not exhibit significant modulatory effects upon mTORC1 activity under any of the conditions tested. While EGF-mediated mTORC1 activation did not immediately influence AR expression **(Fig. 5B)**, long-term exposure for 16-24 hours markedly diminished AR levels **(Fig. 5C)**. Taken together, these data demonstrate that in this hormone-dependent neoplasm, androgen and growth factors independently activate mTORC1 and have additive effects. And, in a feedback manner, mTORC1 suppresses AR levels.

The same experiment was performed with 22R*v1* in order to examine these parameters in the context of an AI model and on a wild type PTEN background. Androgen induced S6K phosphorylation in complete or serum-free medium, albeit to a lower level than that of LNCaP **(Figure 5D)**. In contrast to the results with LNCaP, the highest levels of EGF- and HRG-

induced S6K1 phosphorylation were reached in the absence of androgen and R1881 had either no effect or reduced it in complete or serum-free medium, respectively. The 22R*v1* cell line has an exon 3 duplication mutation (E3DM) that results in the translation of an extended full-length protein (AR-E3DM) and truncated variant lacking the ligand-binding domain (ARΔLBD). We wanted to determine if the expression and ratios of the two AR forms were influenced by androgen and/or growth factors. As expected, R1881 enhanced expression of the full-length protein in both types of media (complete and serum-free). EGF and HRG-β1 increased AR-E3DM expression in complete medium, but decreased it under serum-free conditions. Interestingly, both growth factors markedly increased expression of ARΔLBD in complete medium and generally irrespective of androgen status. Most notably, EGF selectively increased ARΔLBD levels in the absence of androgen and decreased it significantly in the presence of R1881. Although EGF strongly induced MAPK/ERK activation, the differential expression of the truncated form correlated with androgen deprivation and produced a dramatic shift in the ratio of ARΔLBD:AR-E3DM in favor of the truncated form.

DISCUSSION (draft)

The results of this study demonstrate that mTORC1 activity integrates the responses of androgen-sensitive LNCaP cells to androgen withdrawal. Specifically, our data demonstrate that mTORC1 activity is down-regulated in the absence of androgen and can be reactivated upon its re-addition. There is also strong evidence that implicates PI3K-Akt hyperactivation in further suppressing AR signaling after removal of androgen; this highlights the existence of a tightly regulated PI3K-AR signaling axis or loop in which each component negatively regulates or controls the other. Furthermore, the transition to androgen independence is characterized by a dissociation of the negative regulatory effects. Although it is difficult to construct a hierarchy of the androgen withdrawal-induced biochemical events, we can discuss them as they might occur in response to androgen ablation. While AR acts as an upstream negative regulator of PI3K, it is also a direct target for phosphorylation by Akt at Ser210 and Ser790 **(23)**. Paradoxically, Akt phosphorylation mediates down-regulation of AR transactivation and might contribute to its destabilization in the absence of androgen as a result of phosphorylation-dependent ubiquitylation and proteasomal degradation **(24)**. Indeed, we have observed that Akt immunoprecipitated from androgen-deprived cells can phosphorylate recombinant AR and that MG-132 can partially stabilize AR during androgen withdrawal. While this might be paradoxical, it might be necessary for development of androgen independence since AR signaling can inhibit growth and induce apoptosis in cells hyper-responsive to androgen **(16,23,25)**. However, consistent with the above findings, Akt activation stimulates AR signaling in late passage LNCaP **(26)**.

Recent findings support a hypothesis that mammalian target of rapamycin (mTOR) acts as a critical sensor of androgen signaling and regulator of androgen withdrawal-induced AR down-regulation and PI3K-Akt hyperactivation. First, activation of the rapamycin-sensitive raptor/mTOR complex (mTORC1) by polypeptide growth factors (including EGF, TGF-a, HB-EGF, and IGF-1) has been demonstrated to decrease AR levels via suppression of AR mRNA translation **(27)**. Secondly, in the context of a hormone-dependent neoplasm such as LNCaP, androgen strongly stimulates, and is required to maintain full, basal activation of mTORC1 **(28)**, thereby establishing an AR-mTOR negative feedback regulatory loop. Accordingly, inhibition of mTORC1 with rapamycin or LY294002 abrogates the effects of growth factors and increases basal AR levels in the presence of androgen **(27,29)**. Although androgen withdrawal results in a substantial reduction in mTORC1 activity, the residual mTORC1 signal derived from serum growth factors and nutrients continues to drive down AR expression. The significance of this is underscored by the ability of rapamycin to restore AR expression in androgen-deprived cells to levels equivalent to, or greater than that found under basal, androgen-supplemented conditions. mTORC1 down-regulation also provides an explanation for androgen withdrawal-mediated hyperactivation of the PI3K-Akt pathway. In L6 myocytes, amino acid-stimulated mTOR activity can suppress insulin-mediated PI3K and Akt activation as a result of mTOR/p70S6K1-mediated phosphorylation of insulin receptor substrate-1 (IRS-1) **(30-32)**. Although we are speculating that androgen attenuates PI3K through a similar mechanism in LNCaP cells, we have observed that *1)* rapamycin treatment recapitulates the elevation of Akt Ser473 phosphorylation observed in response to androgen withdrawal and *2)* that further treatment of androgen-ablated cells does not increase phospho-Akt levels further. Importantly, the ability of rapamycin to augment two dominant survival signaling pathways (*i.e.*, AR, PI3K-Akt) by rapamycin, suggest the use of mTOR inhibitors for the treatment of PTEN mutant CaPs should be carefully considered since these are common features of AI CaP cells.

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ABBREVIATIONS

AI, androgen-independent; AD, androgen-depleted; AR, androgen receptor; ARE, androgen response element; ARR, androgen response region; AW, androgen withdrawal; CaP, carcinoma of the prostate; CDT-FBS, charcoal/dextran-treated-FBS; DBD, DNA binding domain; FBS, fetal bovine serum; GAPD, glyceraldehyde 3-dehydrogenase; IGF-1, insulin-like growth factor-1; IR, ionizing radiation; NE, neuroendocrine; NED, neuroendocrine differentiation; NSE, neuron-specific enolase; PI3K, phosphatidylinositol 3-kinase; PSA, prostate-specific antigen; RNAi, RNA interference; siRNA, small interfering RNA.

FIGURE LEGENDS

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